(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 400 246 A1**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: **89401499.2**

(51) Int. Cl.5: **C12N 15/32, A01N 63/00**

(22) Date of filing: **31.05.89**

(43) Date of publication of application:
05.12.90 Bulletin 90/49

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(71) Applicant: **PLANT GENETICS SYSTEMS, N.V.**
Kolonel Bourgstraat 106 Bus 1
B-1040 Brussel(BE)

(72) Inventor: **Van Mellaert,Herman**
Wilselsesteenweg 19
B-3200 Leuven(BE)
Inventor: **Botterman Johan**
Het Wijngaardeke 5
B-9721 Zevergem-De Pinte(BE)
Inventor: **Van Rie,Jeroen**
Gravin Johannalaan 10
B-9900 Eeklo(BE)
Inventor: **Joos Henk**
Oostmolen Zuid 5
B-9880 Aalter(BE)

(74) Representative: **Grosset-Fournier, Chantal Catherine et al**
SC Ernest Gutmann/Yves Plasseraud 67 boulevard Haussmann
F-75008 Paris(FR)

(54) Prevention of Bt resistance development.

(57) Plants made resistant to insects by transforming their nuclear genome with two or more DNA sequences, each encoding a different non-competitively binding B. thuringiensis protoxin or insecticidal part thereof, preferably the toxin thereof.

EP 0 400 246 A1

PREVENTION OF Bt RESISTANCE DEVELOPMENT

This invention relates to plant cells and plants, the genomes of which are transformed to contain at least two genes, each coding for a different non-competitively binding Bacillus thuringiensis ("B.thuringiensis" or "Bt") insecticidal crystal protein ("ICP") for a specific target insect species, preferably belonging to the order of Lepidoptera or Coleoptera. Such transformed plants have advantages over plants transformed with a single B. thuringiensis ICP gene, especially with respect to the prevention of resistance development in the target insect species against the at least two B. thuringiensis ICPs, expressed in such plants.

This invention also relates to a process for the production of such transgenic plants, taking into account the competitive and non-competitive binding properties of the at least two B. thuringiensis ICPs in the target insect species' midgut. Simultaneous expression in plants of the at least two genes, each coding for a different non-competitively binding B. thuringiensis ICP in plants, is particularly useful to prevent or delay resistance development of insects against the at least two B. thuringiensis ICPs expressed in the plants.

This invention further relates to a process for the construction of novel plant expression vectors and to the novel plant expression vectors themselves, which contain at least two B. thuringiensis ICP genes encoding at least two non-competitively binding B. thuringiensis ICPs. Such vectors allow integration and coordinate expression of the at least two B. thuringiensis ICP genes in plants.

BACKGROUND OF THE INVENTION

Since the development and the widespread use of chemical insecticides, the occurrence of resistant insect strains has been an important problem. Development of insecticide resistance is a phenomenon dependent on biochemical, physiological, genetic and ecological mechanisms. Currently, insect resistance has been reported against all major classes of chemical insecticides including chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroid compounds (Brattsten et al., 1986).

In contrast to the rapid development of insect resistance to synthetic insecticides, development of insect resistance to bacterial insecticides such as B. thuringiensis sprays has evolved slowly despite many years of use (Brattsten et al. .1986). The spore forming gram-positive bacterium B. thuringiensis produces a parasporal crystal which is composed of crystal proteins (ICPs) having insecticidal activity. Important factors decreasing the probability of emergence of resistant insect strains in the field against B. thuringiensis sprays are firstly the short half life of B. thuringiensis after foliar application, secondly the fact that commercial B. thuringiensis preparations often consist of a mixture of several insecticidal factors including spores, ICP(s) and eventually beta-exotoxin (Shields, 1987) and thirdly the transitory nature of plant-pest interactions. Many successful field trials have shown that commercial preparations of B. thuringiensis containing the spore-crystal complex, effectively control lepidopterous pests in agriculture and forestry (Krieg and Langenbruch,1981). B. thuringiensis is at present the most widely used pathogen for microbial control of insect pests. Various laboratory studies in which selection against B. thuringiensis was applied over several generations of insects have confirmed that resistance against B. thuringiensis is seldom obtained. However, it should be emphasized that the laboratory conditions represented rather low selection pressure conditions (cfr.infra).

For example, Goldman et al. (1986) have applied selection with B. thuringiensis israelensis toxin over 14 generations of Aedes aegypti and found only a marginal decrease in sensitivity. The lack of any observable trend toward decreasing susceptibility in the selected strains may be a reflection of the low selection pressure (LC_{50}) carried out over a limited number of generations. However, it should be pointed out that Georghiou et al. (In : Insecticide Resistance in Mosquitoes : Research on new chemicals and techniques for management. In "Mosquito Control Research, Annual Report 1983, University of California.") with Culex quinquefasciatus obtained an 11-fold increase in resistance to B. thuringiensis israelensis after 32 generations at LC_{95} selection presssure.

McGaughey (1985) reported that the grain storage pest Plodia interpunctella developed resistance to the spore-crystal complex of B. thuringiensis : after 15 generations of selection with the Indian meal moth, Plodia interpunctella, using a commercial B. thuringiensis preparation (HD-1, Dipel. Abbott Laboratories. North Chicago, Illinois 60064, USA) a 100 times decrease in B. thuringiensis sensitivity was reported. Each of the colonies was cultured for several generations on a diet treated with a constant B. thuringiensis dosage which was expected to produce 70-90% larval mortality. Under these (high selection presssure) conditions insect resistance to B. thuringiensis progressed rapidly. More recently, development of resistance against B. thuringiensis is also reported for the almond moth, Cadra cautella (McGaughey and

Beeman, 1988). Resistance was stable when selection was discontinued and was inherited as a recessive trait (McGaughey and Beeman, 1988). The mechanism of insect resistance to B. thuringiensis toxins of Plodia interpunctella and Cadra cautella has not been elucidated. The main cause of B. thuringiensis resistance development in both cases were the environmental conditions prevailing during grain storage. Under these conditions the environment is relatively stable, so B. thuringiensis degradation is slow and permits successive generations of the pest to breed in the continuous presence of the microbial insecticide. The speed at which Plodia developed resistance to B. thuringiensis in this study, suggests that it could do so within one single storage season in the bins of treated grain.

Although insect resistance development against B. thuringiensis has mostly been observed in laboratory and pilot scale studies, very recent indications of B. thuringiensis resistance development in Plutella xylostella populations in the (cabbage) field have been reported (Kirsch and Schmutterer, 1988). A number of factors have led a continuous exposure of P. xylostella to B. thuringiensis in a relatively small geographic area. This and the short generation cycle of P. xylostella have seemingly led to an enormous selection pressure resulting in decreased susceptibility and increased resistance to B. thuringiensis.

A procedure for expressing a B. thuringiensis ICP gene in plants in order to render the plants insect-resistant (EP patent publication 0193259 [which is incorporated herein by reference]; Vaeck et al., 1987; Barton et al., 1987: Fischhoff et al., 1987) provides an entirely new approach to insect control in agriculture which is at the same time safe, environmentally attractive and cost-effective. An important determinant for the success of this approach will be whether insects will be able to develop resistance to B. thuringiensis ICPs expressed in transgenic plants (Vaeck et al., 1987; Barton et al., 1987; Fischhoff et al., 1987). In contrast with a foliar application after which B. thuringiensis ICPs are rapidly degraded, the transgenic plants will exert a continuous selection pressure. It is clear from laboratory selection experiments that a continuous selection pressure has led to adaptation to B. thuringiensis and B. thuringiensis components in several insect species (cfr.supra). In this regard, it should be pointed out that the conditions in the laboratory which resulted in the development of insect-resistance to B. thuringiensis are very well comparable to the situation in transgenic plants which will produce B. thuringiensis ICPs and provide a continuous selection pressure on insect populations feeding on said plants. Mathematical models of selection pressure predict that if engineered insect-resistant plants become a permanent part of their environment, resistance development in insects will emerge rapidly (Gould, 1988). Thus the chances for the development of insect resistance to B. thuringiensis in transgenic plants may be considerably increased as compared to the field application of B. thuringiensis sprays. A Heliothis virescens strain has been reported that is 20 x resistant to B. thuringiensis HD-1 ICP produced by transgenic Pseudomonas fluorescens and 6x resistant to pure ICP (Stone et al., 1989). Furthermore, the monetary and human costs of resistance are difficult to assess, but loss of pesticide effectiveness invariably entails increased application frequencies and dosages and, finally, more expensive replacement compounds, as new pesticides become more difficult to discover and develop. Therefore it is desirable to develop strategies that can greatly delay or prevent the evolution of resistance to B. thuringiensis.

B. thuringiensis strains active against Lepidoptera (Dulmage et al., 1981), Diptera (Goldberg and Margalit, 1977) and Coleoptera (Krieg et al., 1983) have been described. It has become clear from various publications that there is a substantial heterogeneity among ICPs from different strains active against Lepidoptera as well as among ICPs from strains active against Coleoptera. An overview of the different B. thuringiensis ICP genes that have been characterized is given in table 1.

Most of the anti-Lepidopteran crystal protein genes encode 130 to 140 kDa protoxins which dissolve in the alkaline environment of the insect midgut and are proteolytically activated into an active toxin of 60-65 kDa. (Bt3, Bt2, Bt73, Bt14, Bt15, Bt4, Bt18). All these toxins are related and can be recognized as members of the same family based on sequence homologies, the sequence divergence however is substantial and also the insecticidal spectrum among the order Lepidoptera may be substantially different (Höfte et al., 1988).

The P2 toxin gene and the cry B2 gene are more divergent from the above mentioned genes in that they do not encode high molecular weight protoxins but rather toxins of around 70 kDa (Donovan et al., 1988; Widner and Whiteley, 1989, respectively).

It has recently become clear that heterogeneity exists also in the anti-Coleopteran toxin gene family. Whereas several previously reported toxin gene sequences from different B. thuringiensis isolates with anti-Coleopteran activity were identical (European patent ("EP") publications 0149162 and 0202739), the sequences and structure of bt21 and bt22 are substantially divergent (EP patent application n° 89400428.2).

While the insecticidal spectra of ICPs are different, the major pathway of their toxic action is believed to be common. All B. thuringiensis ICPs, for which the mechanism of action has been studied in any detail,

interact with the midgut epithelium of sensitive species and cause lysis of the epithelial cells (Knowles and Ellar, 1986) due to the fact that the permeability characteristics of the brush border membrane and the osmotic balance over this membrane are perturbed. In the pathway of toxic action of B. thuringiensis ICPs, the binding of the toxin to receptor sites on the brush border membrane of these cells is an important feature (Hofmann et al., 1988b). The toxin binding sites in the midgut can be regarded as an ICP-receptor since toxin is bound in a saturable way and with high affinity (Hofmann et al., 1988a).

Although this outline of the mode of action of ICPs is generally accepted, it remains a matter of discussion what the essential determinant(s) are for the differences in their insecticidal spectra. Haider et al. (1986) emphasize the importance of specific proteases in the insect midgut. Hofmann et al. (1988b) indicate that receptor binding is a prerequisite for toxic activity and describe that Pieris brassicae has two distinct receptor populations for two toxins. Other authors have suggested that differences in the environment of the midgut (e.g. pH of the midgut) might be crucial.

SUMMARY OF THE INVENTION

In accordance with this invention, a plant is provided having, stably integrated into its genome, at least two B. thuringiensis ICP genes, encoding at least two non-competitively binding insecticidal B. thuringiensis ICPs, preferably the active toxins thereof, against a specific target insect, preferably against Lepidoptera or Coleoptera. Such a plant is characterized by the simultaneous expression of the at least two non-competitively binding B. thuringiensis ICPs.

Also in accordance with this invention, at least two ICP genes, particularly two genes or parts thereof coding for two non-competitively binding anti-Lepidopteran or anti-Coleopteran B. thuringiensis ICPs, are cloned into a plant expression vector. Plant cells transformed with this vector are characterized by the simultaneous expression of the at least two B. thuringiensis ICP genes. The resulting transformed plant cell can be used to produce a transformed plant in which the plant cells: 1) contain the at least two B. thuringiensis ICP genes or parts thereof encoding at least two non-competitively binding anti-Lepidopteran or anti-Coleopteran B. thuringiensis ICPs as a stable insert into their genome; and 2) express the genes simultaneously, thereby conferring on the plant improved insect resistance which is particularly useful to prevent or delay development of resistance to B. thuringiensis of insects feeding on the transformed plant.

Further in accordance with this invention, plant expression vectors are provided which allow integration and simultaneous expression of at least two ICP genes in a plant cell and which comprise one or more chimeric genes, each containing in the same transcriptional unit: a promoter which functions in plant cells to direct the synthesis of mRNA molecules; one or more different ICP genes each encoding a non-competitively binding B. thuringiensis ICP; preferably a marker gene; a 3' non-translated DNA sequence which functions in plant cells for 3' end formation and the addition of polyadenylate nucleotides to the 3' end of the mRNA sequence; and optionally a DNA sequence encoding a protease-sensitive protein part between any two ICP genes.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, "ICP" should be understood as an intact protein or a part thereof which has insecticidal activity and which can be produced in nature by B. thuringiensis. An ICP can be a protoxin, as well as an active toxin or another insecticidal truncated part of a protoxin which need not be crystalline and which need not be a naturally occurring protein.

As used herein, "protoxin" should be understood as the primary translation product of a full-length gene encoding an ICP.

As used herein, "toxin", "toxic core" or "active toxin" should all be understood as a part of a protoxin which can be obtained by protease (e.g., by trypsin) cleavage and has insecticidal activity.

As used herein, "gene" should be understood as a naturally-occurring full-length DNA sequence encoding a protein, such as is found in nature, as well as a truncated fragment thereof encoding the active part of the protein encoded by the full-length DNA sequence.

As used herein, "truncated B. thuringiensis gene" should be understood as a fragment of a full-length B. thuringiensis gene which still encodes at least the toxic part of the B. thuringiensis ICP, preferentially the

toxin.

As used herein, "marker gene" should be understood as a gene encoding a selectable marker (e.g. encoding antibiotic resistance) or a screenable marker (e.g. encoding a gene product which allows the quantitative analysis of transgenic plants).

Two ICPs are said to be "competitively binding ICPs" for a given insect species when one ICP competes for all ICP receptors of the other ICP, which receptors are present in the brush border membrane of the midgut of the insect species.

Two ICPs are said to be "non-competitively binding ICPs" when, for at least one target insect species, the first ICP has at least one receptor for which the second ICP does not compete and the second ICP has at least one receptor for which the first ICP does not compete, which receptors are present in the brush border membrane of the midgut of the insect species.

A "receptor" should be understood as a molecule which can bind to a ligand (here an B. thuringiensis ICP, preferably a toxin) with affinity (typically a dissociation constant (Kd) between 10^{-11} and 10^{-6}M) and saturability. A determination of whether two ICPs are competitively or non-competitively binding ICPs can be made by determining whether: 1) a first ICP competes for all of the receptors of a second ICP when all the binding sites of the second ICP with an affinity in the range of about 10^{-11} to 10^{-6}M can be saturated with the first ICP in concentrations of the first ICP of about 10^{-5}M or less (e.g., down to about 10^{-11}M); and 2) the second ICP competes for the all of the receptors of the first ICP when all the binding sites of the first ICP with an affinity in the range of about 10^{-11} to 10^{-6}M can be saturated with the second ICP in concentrations of the second ICP of about 10^{-5}M or less.

General Procedures

This section describes in broad terms a general procedure for the evaluation and exploitation of at least two B. thuringiensis ICP genes for prevention of resistance development in insects against B. thuringiensis ICPs expressed in transgenic plants. A non-exhaustive list of consecutive steps in the experimental procedure is described, whereas particular examples which are based on this methodology and which illustrate the invention are given in the next section.

In accordance with this invention, the respective ICPs can be isolated in a conventional manner from the respective strains as listed in table 1. The ICPs can be used to prepare monoclonal or polyclonal antibodies specific for these ICPs in a conventional manner (Höfte et al., 1988).

The ICP genes can be isolated from their respective strains in a conventional manner. Preferably, the ICP genes are each identified by digesting total DNA from their respective strains with suitable restriction enzyme(s); size fractionating the DNA fragments, so produced, into DNA fractions of 5 to 10 Kb; ligating such fractions to suitable cloning vectors e.g. pEcoR251, deposited at DSM under the provisions of the Budapest Treaty, accession number n° 4711 on July 13, 1988; transforming E.coli with the cloning vectors and screening the clones with a suitable DNA probe. The DNA probe can be constructed : 1) from a highly conserved region which is commonly present in different B. thuringiensis genes which encode crystal protoxins against Coleoptera or Lepidoptera such as on the basis of the N-terminal amino acid sequence determined by gas-phase sequencing of the purified proteins (European patent application 88/402,115.5).

Alternatively, the desired fragments, prepared from total DNA of the respective strains, can be ligated in suitable expression vectors (e.g. a pUC vector, Yanisch-Perron et al., 1985, with the insert under control of the lac promoter) and transformed in E. coli, and the clones can then be screened by conventional colony immunoprobing methods (French et al., 1986) for expression of the toxins with monoclonal or polyclonal antibodies raised against the toxins produced by said strains.

The isolated B. thuringiensis ICP genes can then be sequenced in a conventional manner according to procedures well-known in the art (e.g. Maxam and Gilbert, 1980).

At present, several ICP genes have been cloned from different subspecies of B. thuringiensis (table 1). The nucleotide sequence from several of these B. thuringiensis ICP genes have been reported. Whereas several sequences are identical or nearly-identical and represent the same gene or slight variants of the same gene, several sequences display substantial heterogeneity and show the existence of different B. thuringiensis ICP gene classes. Several lines of evidence suggest that all these genes specify a family of related insecticidal proteins. Analysis of the distribution of B. thuringiensis ICPs in different B. thuringiensis strains by determination of the protein composition of B. thuringiensis crystals, by immunodetection using polyclonal antisera or monoclonals against purified crystals, or by use of gene-specific probes, shows that subspecies of B. thuringiensis might contain up to three related B. thuringiensis ICP genes belonging to different classes (Kronstad et al., 1983).

To express the isolated and characterized gene in a heterologous host for purification and characterization of the recombinant protein, the organism of choice is preferentially Escherichia coli. A number of expression vectors for enhanced expression of heterologous genes in E. coli have been described (e.g. Remaut et al., 1981). Usually the gene is cloned under control of a strong regulatable promoter e.g. the lambda pL or pR promoters (e.g. Botterman and Zabeau, 1987), or the lac (e.g. Fuller, 1982) and tac (e.g. De Boer et al., 1983) promoters and provided with suitable translation initiation sites (e.g. Stanssens et al, 1985). Gene cassettes of the B. thuringiensis ICP genes can be generated by site directed mutagenesis, e.g. according to the procedure described by Stanssens et al., 1985. This allowed to create cassettes comprising for example truncated gene fragments encoding the toxin core fragment or hybrid genes with a selectable marker according to the procedures as described in patent application EP 88402241.9.

The cells of an E. coli culture, which has been induced to produce the recombinant ICPs, are harvested. The method to induce the cells to produce the recombinant toxin depends on the choice of the promoter (e.g. lac (Fuller, 1982) is induced by isopropyl-B-D-thiogalacto-pyranoside (IPTG); pL is induced by temperature shock (Bernard et al., 1979)). The recombinant ICP is usually deposited in the cells as insoluble inclusions (Hsuing and Becker, 1988). The cells are lysed to liberate the inclusions. The bulk of E. coli proteins is removed in subsequent washing steps. A semi-purified protoxin pellet is obtained, the protoxin can be dissolved in alkaline buffer (e.g. Na_2CO_3, pH 10). The procedure for Bt2, which is also applicable to other recombinant toxins, has been described by Höfte et al., 1986.

In accordance with this invention the binding of various toxins to ICP receptors on the brush border membrane of the columnar midgut epithelial cells of various insect species is investigated. The brush border membrane is the primary target of the toxin, and membrane vesicles preferentially derived from the brush border membrane can be obtained according to Wolfersberger et al., 1987.

The binding to ICP receptors of one or more ICPs (e.g., ICP A, ICP B, etc.) is usually characterized as follows (Hofmann et al, 1988b):

1. ICP A is labelled with a suitable marker (usually a radioisotope such as ^{125}I)
2. Brush border membranes are incubated with a small amount (preferably less than 10^{-10} M) of labelled ICP A together with different concentrations of non-labelled ICP A (preferably from less than 10^{-11} to 10^{-5} M)
3. For all concentrations tested the amount of labelled ICP A bound to the brush border membranes is measured.
4. Mathematical analysis of these data allows one to calculate various characteristics of the ICP receptor such as the magnitude of the population of binding sites (Scatchard, 1949).
5. Competition by other toxins (e.g. toxin B) is preferably studied by incubating the same amount of labelled ICP A with brush border membranes in combination with different amounts of ICP B (preferentially from 10^{-11} to 10^{-6} M). Subsequently, steps 3 and 4 are repeated.

By this procedure, it has been found that Bt3 toxin, Bt2 toxin and Bt73 toxin are competitively binding anti-Lepidopteran ICPs for Manduca sexta and Heliothis virescens (see example 6). Various other combinations of toxins have been found to be non-competitively binding anti-Lepidopteran or anti-Coleopteran toxins (example 6). These combinations are given as examples and do not limit the scope of the invention.

Although, the concept of competitivity versus non-competitivity of ICP binding does not have any immediate practical importance by itself, the observation of the non-competitivity of two B. thuringiensis ICPs active against the same target insect is of practical importance because such a combination of two non-competitively binding B. thuringiensis ICPs can be used to prevent resistance development of in the target insect against such B. thuringienis ICPs. A selection experiment with M. sexta, using Bt2, Bt18, and a mixture of Bt2 and Bt18 toxins, has shown that Bt2 and Bt18 are two non-competitively binding anti-Lepidopteran toxins. After 20 generations of selection, a very pronounced reduction in ICP sensitivity was observed in the selection experiments with Bt2 or Bt18 alone (>100 times). The reduction in sensitivity in the selection experiment with a Bt2-Bt18 mixture was only marginal (3 times).

This demonstrates the unexpected advantage of a simultaneous use of two non-competitively binding ICPs, in a situation which models the high selection pressure which will exist with the use of transgenic plants. The two resistant strains selected respectively with Bt2 and Bt18 showed a specific loss in receptor sites for respectively Bt2 and Bt18 toxin. In each case, receptor sites for the toxin which was not used for selection were not affected or their concentration even increased. Thus, the Bt2 selected strain retained its Bt18 receptors, and the Bt18 selected strain developed an increased number of Bt2 receptors. Indeed, the Bt18 selected strain showed an increased sensitivity for Bt2 along with its increased Bt2 receptor concentration. No significant changes in receptor sites were found in the strain selected against the combined toxins. These findings are described in detail in example 7. This finding has immediate relevance to any combination of non-competitively binding anti-Lepidopteran or Coleopteran ICPs which will have the

same beneficial effect against their common target insects.

Such a combination, when directly expressed in the transgenic plant, will also offer the substantial advantage of reducing the chance for development of insect resistance against the B. thuringiensis ICPs expressed in the plant. There may be additional benefits because the combined spectrum of two toxins may be broader than the spectrum of a single ICP expressed in a plant (e.g. examples 8, 9 and 10).

If, among two competitively binding ICPs, one has a larger binding site population than the other against a given target insect, it will be most advantageous to use the one with the largest population of binding sites to control this target pest in a combination with the most suitable non-competitively binding B. thuringiensis ICP. For example, as described in example 6, it will be useful to use Bt73 against Heliothis virescens rather than Bt2 or Bt3, and it is preferred to use Bt3 against Manduca sexta rather than Bt2 or Bt73. The selected gene can then be combined with the best suitable non-competitively binding ICP.

Previously, plant transformations involved the introduction of a marker gene together with a single ICP gene, within the same plasmid, in the plant genome (e.g. Vaeck et al., 1987; Fischoff et al., 1987). Chimeric ICP genes usually comprised either an intact ICP gene or a truncated ICP gene fragment encoding the toxin core or hybrid gene with a selectable marker gene such as neo coding for neomycin phosphotransferase. The chimeric ICP and any chimeric marker gene were between the T-DNA border repeats for A. tumefaciens Ti-plasmid mediated transformation.

This invention involves the combined expression of two or even more B. thuringiensis ICP genes in transgenic plants. The insecticidally effective B. thuringiensis ICP genes, which encode two non-competitively binding ICPs for a target insect species, preferably the respective truncated ICP genes, are inserted in a plant cell genome, preferably its nuclear genome, so that the inserted genes are downstream of, and under the control of, a promoter which can direct the expression of the genes in the plant cell. This is preferably accomplished by inserting in the plant cell genome one or more chimaeric genes, each containing in the same transcriptional unit: at least one ICP gene; preferably a marker gene; and optionally a DNA sequence encoding a protease (e.g., trypsin) sensitive or cleavable protein part between any two ICP genes in a chimaeric gene. Each chimaeric gene also contains a promoter which can direct the at least one ICP gene in the plant cell.

Preferred promoters for such chimaeric genes include: the strong constitutive 35S promoter obtained from the cauliflower mosaic virus, isolates CM 1841 (Gardner et al., 1981), CabbB-S (Franck et al., 1980) and CabbB-JI (Hull and Howell, 1987); and the TR1′ promoter and the TR2′ promoter which drive the expression of the 1′ and 2′ genes, respectively, of the T-DNA (Velten et al., 1984). Alternatively, a promoter can be utilized which is specific for one or more tissues or organs of the plant whereby the inserted genes are expressed only in cells of the specific tissue(s) or organ(s). Examples of such promoters are a stem-specific promoter such as the AdoMet-synthetase promoter (Peleman et al., 1989) or a a seed-specific promoter such as the 2S promoter (Krebbers et al., 1988). For example, the genes could be selectively expressed in the leaves of a plant (e.g., potato) by placing the genes under the control of a light-inducible promoter such as the promoter of the ribulose-1,5-bisphosphate carboxylase small subunit gene of the plant itself or of another plant such as pea as disclosed in U.S. patent application 821,582 and European patent application 86/300, 291.1. Another alternative is to use a promoter whose expression is inducible (e.g. by temperature or chemical factors).

Finally, a 3′ non-translated DNA sequence, which functions in plant cells for 3′ end formation and the addition of polyadenylate nucleotides to the 3′ end of the mRNA sequence encoded by the at least one ICP gene in the plant cell, also forms part of each of such chimeric genes. Examples are the 3′ untranslated end of the octopine synthase gene, the nopaline synthase gene and the T-DNA gene 7 (Velten and Schell, 1985).

Using A. tumefaciens Ti vector-mediated transformation methodology, all chimeric genes are between the T-DNA border repeats of suitable disarmed Ti-plasmid vectors (Deblaere et al., 1988). The chimeric genes can also be in non-specific plasmid vectors which can be used for direct gene transfer (e.g. as described by Pazkowski et al., 1984; De La Pena et al., 1986). Different strategies can be followed to obtain a combined expression of two B.thuringiensis ICP genes in transgenic plants as summarized below:

I Chimeric gene constructs whereby two or more ICP genes and a marker gene are transferred to the plant genome as a single piece of DNA and lead to the insertion in a single locus in the genome

Ia The genes can be engineered in different transcriptional units each under control of a distinct promoter

To express two or more ICP genes and a marker gene as separate transcriptional units, several promoter fragments directing expression in plant cells, can be used as mentioned earlier. All combinations of these promoters mentioned above in the chimaeric constructs for one ICP gene are possible. Examples of such individual chimeric constructs are described for the bt2 gene in EP patent publication EP 0193259, for the bt13 gene in EP patent application n° 88402115.5 and for the bt18 gene in Ep patent application n° 88402241.9. The ICP genes in the chimeric genes of this invention can be the intact ICP gene or a truncated gene fragment encoding the toxic core of the ICP. The individual chimeric genes are cloned in the same plasmid vector according to standard procedures.

Ib Two genes (e.g., either an ICP and a marker gene or two ICP genes) or more can be combined in the same transcriptional unit

To express two or more ICP genes in the same transcriptional unit, the following cases can be distinguished:

In a first case, hybrid genes in which the coding region of one gene is in frame fused with the coding region of another gene can be placed under the control of a single promoter. Fusions can be made between either an ICP and a marker gene or between two ICP genes. An example of an ICP gene-marker gene fusion has been described in EP patent publication 0193259, i.e. a hybrid bt2-neo gene encoding a Bt2-NPTII fusion protein was used.

Another possibility is the fusion of two ICP genes. Between the genes, each encoding an ICP which still is insecticidally active (i.e., a toxic part of the protoxin), a gene fragment encoding a protease (e.g., trypsin) sensitive protein part should be included, such as a gene fragment encoding a part of the N-terminal or C-terminal amino acid sequence of one of the ICPs which is removed or cleaved upon activation by the midgut enzymes of the target insect species.

In a second case, the coding regions of the respective genes can be combined in dicistronic units placed under the control of a promoter. The coding regions of the two ICP genes are placed after each other with an intergenic sequence of defined length. A single messenger RNA molecule is generated leading to the translation into two separate gene products. Based on the modified scanning model (Kozak, 1987), the concept of reinitiation of translation has been accepted provided that a termination codon in frame with the upstream ATG precedes the downstream ATG. Experimental also demonstrated that the plant translational machinery is able to synthesize several polypeptides from a polycistronic mRNA (Angenon et al., in preparation).

II Chimeric constructs with one or more ICP genes that are transferred to the genome of a plant already transformed with a one or more ICP genes

Several genes can be introduced into a plant cell during sequential transformation steps (retransformation) if an alternative system to select transformants is available for the second round of transformation. This retransformation leads to the combined expression of ICP genes which are introduced at multiple loci in the genome. Preferably two different selectable marker genes will be used in the two consecutive transformation steps. The first marker will be used for selection of transformed cells in the first transformation, while the second marker is used for selection of transformants in the second round of transformation. Sequential transformation steps using kanamycin and hygromycin have been described for example by Sandler et al., 1988 and Delauney et al., 1988.

III Chimeric constructs with one or more ICP genes, that are separately transferred to the nuclear genome of separate plants in independent transformation events and are subsequently combined in a single plant genome through crosses.

The first plant should be a plant transformed with a first ICP gene or an F1 plant derived herefrom through selfing (preferably an F1 plant which is homozygous for the ICP gene). The second plant should be a plant transformed with a second ICP gene or an F1 plant derived herefrom through selfing (preferably an F1 plant which is homozygous for the second ICP gene). Selection methods can be applied to the plants obtained from this cross in order to select those plants having the two ICP genes present in their genome (e.g. Southern blotting) and expressing the two ICPs (e.g. separate ELISA detection of the immunologically

different ICPs).

In particular this will be a useful strategy to produce hybrid varieties from two parental lines transformed with a different ICP gene as well as to produce inbred lines containing two different ICP genes through crossing of two independent transformants (or their F1 selfed offspring) from the same inbred line.

IV Chimeric constructs with one or more ICP genes separately transferred to the genome of a single plant in the same transformation experiment leading to the insertion of the respective chimeric genes at multiple loci.

Cotransformation consists in the simultaneous transformation of a plant with two different expression vectors, one containing the first ICP gene, the second one containing the second ICP gene. Along with each ICP gene, a different marker gene may be used and selection can be applied with the two markers simultaneously. Alternatively a single marker can be used and a sufficiently large number of selected plants can be screened again in order to find those plants having the two ICP genes (e.g. by Southern blotting) and expressing the two proteins (e.g. by means of ELISA). Cotransformation with more than one T-DNA has been accomplished by using simultaneously two different strains of Agrobacterium, each with a different Ti-plasmid (Depicker et al., 1985) or with one strain of Agrobacterium containing two T-DNAs on separate plasmids (de Framond et al., 1986). Direct gene transfer using a mixture of two plasmids has been used to cotransform plant cells with a selectable and a non-selectable gene (Schocher et al., 1986).

Transgenic plants obtained can be used in further plant breeding schemes. The transformed plant can be selfed to obtain a plant which is homozygous for the inserted genes. If the plant is an inbred line this homozygous plant can be used to produce seeds directly or as a parental line for a hybrid variety. The gene can also be crossed into open pollinated populations or other inbred lines of the same plant using conventional plant breeding approaches.

It is obvious that other plant transformation methods can be used and are within the scope of the invention as long as the result is a plant which expresses two or more non-competitively binding ICPs. Needless to say, this invention is not limited to the use ofAgrobacterium tumefaciens Ti-plasmids for transforming plant cells with ICP genes encoding such non-competitively binding ICPs. Other known methods for plant cell transformation such as by electroporation or vector systems based on plant viruses or pollen, can be used for transforming monocotyledonous and dicotyledonous plants in order to obtain plants, which express two non-competitively binding ICPs. Furthermore, DNA sequences encoding two non-competitively binding ICPs other than those disclosed herein can be used for transforming plants. It will be clear to those skilled in the art that each of the ICP genes can be encoded by equivalent DNA sequences, taking into consideration the degeneration of the genetic code. Also, ICPs with only few amino-acid changes obtained through mutations in the ICP gene could still encode a protein with essentially the same characteristics (e.g. insecticidal activity, receptor binding).

The following examples illustrate the invention and are not intended to limit its scope. Those skilled in the art will recognize that other combinations of two or more non-competitively binding B. thuringiensis ICP genes can be used to transform plants in order to prevent the development in a target insect of resistance against B. thuringiensis ICPs expressed in transgenic plants and that equivalent embodiments are included herein. All procedures for making and manipulating DNA were carried out by the standardized procedures described in Maniatis et al, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory (1982).

EXAMPLE 1: Collection of genes

The collection of anti-Lepidopteran and anti-Coleopteran Bt genes encoding ICPs, which are the subject of the examples, is described in table 1. References for the respective genes are indicated. The origin, the isolation and characterization of the Bt genes, which have not been published, are described below. Bt strains, such as strains HD-68, HD-110, and HD-73, are publicly available from the Agricultural Research Culture Collection, Norther Region Research Center, North University, Peoria, Illinois 61604, U.S.A.

bt3 gene:

From B. thuringiensis var. aizawai HD-68 a ICP gene was cloned. Characterization of this gene revealed

an open reading frame of 3528 bp which encodes a protoxin of 133 kDa. This gene was identical to the one described by Schnepf et al., 1985.

bt4 gene:

A genomic library was prepared from total DNA of strain B. thuringiensis aizawai HD-68. Using the 1.1 kb internal HindIII fragment of bt2 as a probe, a gene designated bt4 was isolated. Characterization of this gene revealed an open reading frame of 3495 bp which encodes a protoxin of 132 kDa and a trypsin activated toxin fragment of 60 kDa. This (insect controlling protein) gene differs from previously identified genes and was also found in several other strains of subspecies aizawai and entomocidus including HD-110. Fig. 13 shows the nucleotide sequence and deduced amino acid sequence of the open reading frame ("ORF") of the bt4 gene extending from nucleotide 264 to nucleotide 3761.

bt14 and bt15 genes:

A genomic library was prepared from total DNA of strain B. thuringiensis var. entomocidus HD-110 by partial Sau3A digest of the total DNA and cloning in the vector pEcoR251, deposited at DSM under the provisions of the Budapest Treaty, accession number n° 4711 on July 13, 1988. Using monoclonal antibodies (Höfte et al., 1988), at least three structurally distinct ICPs were identified in crystals of B. thuringiensis entomocidus HD-110. These monoclonal antibodies were used to clone the three different ICP genes from this B. thuringiensis strain. One of these genes is the bt4 gene as described above.
The second gene was called "bt15". Fig. 14 shows the nucleotide sequence and deduced amino acid sequence of the ORF of the bt15 gene, isolated from HD-110, extending from nucleotide 234 to nucleotide 3803. The Shine and Dalgarno sequence, preceding the initiation codon is underlined. This gene has an open reading frame of 3567 bp which encodes a protoxin of 135 kDa and a 63 kDa toxin fragment. A similar gene has been described by Honee et al. 1988, isolated fromB. thuringiensis entomocidus 60.5. The bt15 gene differs from the published sequence at three positions : an Ala codon (GCA) is present instead of an Arg codon (CGA) at position 925 and a consecution of a Thr-His codon (ACGCAT) is present instead of a Thr-Asp codon (ACGGAT) at position 1400. The numbers of the positions are according to Honnee et al., 1988. Another similar gene has been described by Sanchis et al. (EP publication 0295156), isolated from B. thuringiensis aizawai 7-29 and entomocidus 6-01. The bt15 gene is different from this published nucleotide sequence at three different places : 1) a Glu codon (GAA) instead of an Ala codon (GCA) at position 700; 2) the sequence TGG, CCA, GCG, CCA instead of TGC, CAG, CGC, CAC, CAT at position 1456 and 3) an Arg codon (CGT) instead of an Ala codon (GCG) at position 2654. The numbers of the positions are according to Sanchis et al (EP publication 0295156).
The third gene isolated was called "bt14". It has an open reading frame of 3621 bp which encodes a 137 kDa protoxin and a 66 kDa activated toxin fragment. A similar gene has been cloned from B.thuringiensis HD-2 (Brizzard and Whiteley, 1988). The bt14 gene differs from the published nucleotide sequence by two nucleotide substitutions : a T instead of a C at position 126, and a C instead of a T at position 448. In the first case, the Ile codon (ATT or ATC) is conserved whereas in the second case the Tyr codon (TAT) is converted to a His codon (CAC). The numbers of the positions are according to Brizzard and Whiteley (1988).

bt2 gene:

Cloning of the bt2 gene is described in EP publication 0193259.
Cloning of the bt18 gene was performed as described in EP application n° 88402241.9.

bt13 gene:

The bt13 gene was cloned as described in EP application n° 88402115.5.

bt21 and bt22 genes:

both genes, encoding Coleopteran-active ICPs, were cloned as described in EP application n° 89400428.2.

EXAMPLE 2 : construction of gene cassettes and expression of bt genes in E.coli

1) bt2, bt18: the construction of bt2 and bt18 gene cassettes has been described in previous EP patent applications n° 86300291.1 and 88402241.9 respectively. Basically, they comprise a truncated gene encoding the toxin core fragment and a hybrid gene consisting of the toxin core fragment in frame fused to the N-terminus of the neo gene.

2) bt14, bt15: as described in EP application n° 88402241.9, gene cassettes for the bt14 and bt15 genes respectively were designed in order to express the genes in E.coli and in plants. First, a NcoI site was introduced at the N-terminus of the genes by site mutagenesis. In the case of the bt15 gene, the substitution of the TT nucleotides immediately in front of the ATG codon into CC yielded a NcoI site overlapping with the ATG initiation codon. This site was introduced using the pMa/c vectors for site directed mutagenesis (Stanssens et al., 1987) and a 28-mer oligonucleotide with the following sequence:
5′-CGGAGGTATTCCATGGAGGAAAATAATC-3′. This yielded the plasmid pVE29 carrying the N-terminal fragment of the bt15 gene with a NcoI site at the ATG initiation codon.

According to Brizzard and Whiteley (1988), the initiation codon of the bt14 gene is a TTG codon. Similarly, a NcoI site was created at this codon for site directed mutagenesis using a 34-mer oligonucleotide with the following sequence :
5′-CCTATTTGAAGCCATGGTAACTCCTCCTTTTATG-3′.
In this case the sequence of the intitiation codon was converted from ATATTGA to ACCATGG. This yielded the plasmid pHW44 carrying the N-terminal fragment of the bt14 gene with a NcoI site at the initiation codon.
In a second step, the genes were reconstructed by ligating the N-terminal gene fragments with a suitable C-terminal gene fragment yielding a bt15 and bt14 gene respectively with a NcoI site at the ATG initiation codon.
To express the bt14 and bt15 genes encoding the protoxin in E. coli, the following constructs were made: pOH50 contains the bt15 gene under control of the lac promoter, pHW67 contains the bt14 gene under control of the tac promoter. Induction of a culture of the strain WK6 carrying the respective plasmids with IPTG yielded an overproduced protein.
As mentioned, the active toxic fragment of the bt15 and bt14 protoxin comprise a 63 and 60 kDa trypsin digest product respectively. Instead of expressing the whole gene, it is also possible to express a toxin encoding gene fragment or derivatives therefrom in plants. To this end, truncated bt14 and bt15 gene fragments were constructed. In order to be able to select transgenic plants producing the ICP gene products, hybrid genes of the truncated gene fragments were also made with the neo gene encoding a selectable marker as described in EP patent publication EP 0193259.

By comparison of the nucleotide sequence of the bt4, bt14 and bt15 genes respectively with the bt2 and bt18 genes respectively, the BclI site could be identified as a suitable site localized downstream of the coding sequence encoding the toxin gene fragment. To construct a truncated gene fragment and a hybrid gene of the latter with the neo gene, the filled BclI site was ligated to the filled EcoRI site of pLKM91 (Höfte et al., 1986) and the filled HindIII site of pLK94 respectively (Botterman and Zabeau, 1987). pLKM91 carries a 5′ truncated neo gene fragment which codes for an enzymatically active C-terminal gene fragment of neo, and pLK94 contains translation stop codons in three reading frames. This yielded the following plasmids: pHW71 carries a bt14 truncated neo-hybrid gene; pHW72 a truncated bt14 gene, pVE34 a truncated-bt15-neo hybrid gene; pVE35 a truncated bt15 gene. In a similar way as described for bt14 and bt15 in this example, and for bt2 and bt18 in previous EP applications, gene cassettes can be constructed for the bt3 and bt4 genes and the genes can be expressed in E.coli.

EXAMPLE 3: Purification of recombinant ICPs

Purification methods for recombinant Bt2 protoxin were described by Höfte et al. (1986). The same procedure can be applied for other recombinant toxins.

EXAMPLE 4: Purification of activated toxins

Solubilized protoxins of Bt2-, Bt3-, Bt4-, Bt14-, Bt15-, Bt13-, Bt21 (in Na_2CO_3 50mM, DTT 10 mM pH = 10) are dialyzed against 0.5 % $(NH_4)_2CO_3$ at pH 8 and treated with trypsin (trypsin:protoxin 1:20 w:w) for 2h at 37° C. The activated toxin is chromatographically purified (Mono-Q column on FPLC) as described by Hofmann et al.(1988b).

EXAMPLE 5: Determination of the insecticidal spectrum

Recombinant protoxins or activated toxins can be evaluated for their insecticidal activity. The protoxin is dissolved in alkaline buffer containing a reducing agent (Na_2CO_3 50 mM, DTT 10 mM pH = 10), or the toxin is used as soluble protein directly from FPLC. Protein concentrations are determined. Subsequently dilutions of the protoxin or toxin solution are prepared in PBS buffer pH = 7.4 containing 0.15 M NaCl and 0.1 % Bovine serum albumin.

Artificial medium for insect culture (e.g. the medium described by Bell and Joachim, 1976 for Manduca sexta) is poured in appropriate recipients and allowed to solidify. Subsequently a quantity of the (pro)toxin dilutions is applied on this medium and the water is allowed to evaporate under a laminar flow. This results in medium with a certain quantity of toxin coated on the surface of the artificial medium. (quantities are usually in the range of 0.1 to 10000 ng.cm2). E.g. for Bt2-toxin typical dilutions for a toxicity test on Manduca sexta are 1,5,25,125 and 625 ng.cm2. First instar larvae of Manduca sexta are then applied on the medium and growth and mortality are assessed after 6 days. Mortality increases with dosage. Dose response data can be analysed in probit analysis (Finney, 1962) and the data are best summarized by an LD50 value, this is the amount of toxin which kills 50 % of the insects. The LD50 for Bt2-toxin against Manduca sexta is around 20 ng.cm2.

Similar assays are possible for other insect species using a suitable diet, or similar setups are also possible applying the toxin on leaves for insects for which no artificial diet is used.

EXAMPLE 6: Binding studies

Toxins

All protoxins and their toxic fragments were purified according to the methods described for the Bt2-protoxin and toxin in Höfte et al. (1986) and EP patent publication EP 0193259. The activated and purified toxins are further referred to as the Bt2-, Bt3-, Bt4-, Bt14-, Bt15-, Bt13-, Bt21- and Bt22-toxins.

It has been shown that B. thuringiensis var. kurstaki HD73 produces one protein of 133 kDa encoded by a 6.6 kb type gene. A culture of this strain was grown as described by Mahillon and Delcour (1984). The autolysed culture was spun down (20 minutes at 4500 rpm in a HB4 rotor) and washed with a buffer containing 20 mM Tris, 100 mM NaCl and 0.05 % Triton X-100, pH 8. The final pellet was resuspended in this buffer (4 ml buffer for 100 ml culture). This solution was then layered onto a linear Urograffin gradient (60-70%) which was centrifuged in a SW 28 rotor for 90 minutes at 18000 rpm. Crystals were collected and stored at -20° C until further use. Activation was performed according to Höfte et al. (1986). The purified toxin is further referred to as the Bt73-toxin.

Iodination of ICPs

Iodination of Bt2-, Bt3-, and Bt73-toxin was performed using the Chloramin-T method (Hunter and Greenwood, 1962). 1 mCi ^{125}I-NaI and 20 to 37.5 ug Chloramin-T in NaCl/P_i were added to 50 ug of purified toxin. After gentle shaking for 60 seconds, the reaction was stopped by adding 53 ug of potassium metabisulfite in H_2O. The whole mixture was loaded on a PD 10 Sephadex G-25M gelfiltration column to remove free iodine. A subsequent run on a Biogel P-60 column was carried out in order to increase the purity. Alternatively, toxins were labeled using the Iodogen method. Iodogen (Pierce) was dissolved in chloroform at 0.1 mg/ml. 100 ul of this solution was pipetted into a disposable glass vessel and dried under a stream of nitrogen gas. The vessel was rinsed with Tris buffer (20 mM Tris, pH 8.65 with 0.15 M NaCl). 50 ug of toxin (in Tris buffer) was incubated with 1 mCi of ^{125}I-NaI in the tube for 10 minutes. The reaction was then stopped by the addition of 1 M NaI (one fourth of the sample volume). The sample was immediately loaded onto a PD10 Sephadex G-25M column and later on a Biogel P-60 column to remove

free iodine and possible degradation products. Other toxins were iodinated using one of the above mentioned procedures.

Determination of specific activity of iodinated toxin

Specific activity of iodinated Bt2-, Bt3-, and Bt73-toxin samples was determined using a 'sandwich' ELISA technique according to Voller, Bidwell and Barlett (1976) in Manual of Clinical Immunology (Rose and Friedman, eds) pp. 506-512, American Society of Microbiology, Washington). Primary antibody was a polyclonal antiserum raised against Bt2-toxin and the secondary antibody was a monoclonal antibody 4D6 (unpublished results).

The conjugate used was alkaline phosphatase coupled to anti mouse IgG. The reaction intensity of a standard dilution series of unlabeled toxin and dilutions of the iodinated toxin sample (in NaCl/P_i - 0.1 % BSA) was measured. Linear regression calculations yielded the protein content of the radioactive toxin sample. The samples with the highest specific activities were used in the binding assays. Specific activities were 59400, 33000 and 19800 Ci/mole (on reference date) for Bt73-toxin (labeled according to Iodogen procedure), Bt2-toxin (Chloramin-T method) and Bt3-toxin (Iodogen method) respectively.

Specific activities of other toxins were determined using a similar approach. Specific monoclonal and polyclonal antibodies for each of these toxins were raised and applied in ELISA.

Preparation of brush border membrane vesicles

Brush border membrane vesicles ("BBMV") from Manduca sexta, Heliothis virescens, Spodoptera littoralis, Plodia interpunctella, Mamestra brassicae, Pieris brassicae, Leptinotarsa decemlineata were prepared according to the method of Wolfersberger et al. (1987). This is a differential centrifugation method that makes use of the higher density of negative electrostatic charges on luminal than on basolateral membranes to separate these fractions.

Binding assay

Duplicate samples of ^{125}I-labeled toxin, either alone or in combination with varying amounts of unlabeled toxin, were incubated at the appropriate temperature with brush border membrane vesicles in a total volume of 100 ul of Tris buffer (Tris 10 mM, 150 mM NaCl, pH 7.4). All buffers contained 0.1 % bovine serum albumin (BSA). The incubation temperature was 20 C. Ultrafiltration through Whatman GF/F glass fiber filters was used to separate bound from free toxin. Each filter was rapidly washed with 5 ml of ice-cold buffer (NaCl/P_i- 0.1 % BSA). The radioactivity of the filter was measured in a gammacounter (1275 Minigamma, LKB). Binding data were analyzed using the LIGAND computer program. This program calculates the bound concentration of ligand as a function of the total concentration of ligand, given the affinity (Ka or its inverse Kd = 1/Ka, the dissociation constant) and the total concentration of receptors or binding site concentration (R_t).

Determination of protein concentration

Protein concentrations of purified Bt2-, Bt3-, Bt73-and Bt15-toxin were calculated from the OD at 280 nm (measured with a Uvikon 810 P, Kontron Instruments spectrofotometer). The protein content of solutions of other toxins and of brush border membrane vesicles (BBMV) as measured according to Bradford (1976).

Binding of Bt2, Bt3 and Bt73 toxin to BBMV of Manduca sexta and Heliothis virescens : an example of 3 competitively binding Lepidopteran ICPs.

Bt2, Bt3 and Bt73 toxins are toxic to both Manduca sexta and Heliothis virescens: LC50 values for Manduca sexta are respectively 20.20 and 9 ng/cm2 ; for Heliothis virescens the LC50's are 7.157 and 9 ng/cm2.

Labelled toxin, either Bt3 (0.8 nM) or Bt2 (1.05 nM) or Bt73 (1.05 nM) was incubated with BBMV in a

volume of 0.1 ml. BBMV protein concentrations were 100 ug/ml for M. sexta and for Bt2-H. virescens, for Bt3-H. virescens 150 and for Bt73-H. virescens 50 ug/ml was used. The labelled toxin was combined with varying amounts of an unlabeled toxin (competitor). After a 30 min incubation bound and free toxin were separated through filtration.

Figs. 1-3 show the percentages binding of respectively labelled Bt2, Bt3 and Bt73 toxin as a function of the concentration of competitor for Manduca sexta. Figs. 4-6 show these data for Heliothis virescens. The amount bound in the absence of competitor is always taken as 100 % binding. Figs. 1-6 show the binding of ^{125}I-labeled toxins to M. sexta (in Figs. 1, 2 and 3) and H. virescens (in Figs. 4, 5 and 6) brush border membrane vesicles ("BBMV"). Vesicles were incubated with labeled toxin [in Figs. 1 and 4: ^{125}I-Bt2-toxin (1.05nM); in Figs. 2 and 5: ^{125}I-Bt3-toxin (0.8nM); in Figs. 3 and 6: ^{125}I-Bt73-toxin (1.05nM] in the presence of increasing concentrations of Bt2-toxin (★), Bt3-toxin (●) or Bt73-toxin (▲). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. On M. sexta vesicles, these amounts were 1820, 601 and 2383 cpm, and on H. virescens vesicles 1775, 472 and 6608 cpm for ^{125}I-Bt2-, Bt3- and Bt73-toxin, respectively. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample.

Figure 1: shows the binding of ^{125}I Bt2 toxin to M. sexta BBMV
Figure 2: shows the binding of ^{125}I Bt3 toxin to M. sexta BBMV
Figure 3: shows the binding of ^{125}I Bt73 toxin to M. sexta BBMV
Figure 4: shows the binding of ^{125}I Bt2 toxin to H. virescens BBMV
Figure 5: shows the binding of ^{125}I Bt3 toxin to H.virescens BBMV
Figure 6: shows the binding of ^{125}I Bt73 toxin to H.virescens BBMV

The conclusions from these figures are that Bt2 and Bt3, Bt3 and Bt73, and Bt2 and Bt73 are competitively-binding ICP's both for Manduca sexta and for Heliothis virescens. Indeed Bt3 competes for the entire population of receptor sites of Bt2 in Manduca sexta (Fig.1): the % labelled Bt2 bound in the presence of 100 nM Bt3 is equal to the % Bt2 bound with 100 nM of Bt2 itself. The opposite is not true: in the presence of 100 nM Bt2 the % of labelled Bt3 is not reduced to the same level as with 100 nM of Bt3 (fig.2).

A similar reasoning is followed to observe competitivity of other toxin combinations : Bt3 competes for the entire population of receptor sites of Bt73 (fig 3) in M. sexta, the opposite is not true (fig 2), Bt2 and Bt73 compete for the entire population of each other's binding sites (Figs. 1 and 3).

In Heliothis virescens : Bt2 competes for the entire population of receptor sites of Bt3 (fig 5), Bt73 competes for the entire population of receptor sites of Bt3 (fig 5) and Bt73 competes for the entire population of receptor sites of Bt2 (Fig. 4), the opposite statements are not true (Figs. 4,5 and 6).

The same data can be used in mathematical analysis (e.g. Scatchard analysis according to Scatchard, 1949 ; analysis with the LIGAND computer program according to Munson and Rodbard, 1980) to calculate the dissociation constant (Kd) of the toxin-receptor complex and the concentration of binding sites (Rt) : the results of these calculations using the LIGAND computer program were the following:

Bt2-M.sexta:	Kd=0.4 nM	Rt=3.4 pmol/mg vesicle protein
Bt3-M. sexta:	Kd=1.5 nM	Rt=9.8 pmol/mg vesicle protein
Bt73-M. sexta:	Kd=0.6 nM	Rt=4.0 pmol/mg vesicle protein
Bt2-H. virescens:	Kd=0.6 nM	Rt=9.7 pmol/mg vesicle protein
Bt3-H. virescens:	Kd=1.2 nM	Rt=3.7 pmol/mg vesicle protein
Bt73-H. virescens:	Kd=0.8 nM	Rt=19.5 pmol/mg vesicle protein

These data demonstrate the high affinity receptor binding of the toxin (Kd's in the range of 10^{-10} to 10^{-9} M.

Binding of Bt2 and Bt14 to BBMV of P. brassicae : an example two non-competitively binding Lepidopteran ICPs

Bt2 and Bt14 toxins are both toxic to P. brassicae. LC50 values were 1.3 and 2.0 ug/ml respectively (5 ul toxin samples were applied on leaf discs fed to first instar P. brassicae larvae). Labelled Bt2 (1.05 nM) or Bt14 (1.4 nM) were incubated with BBMV from P. brassicae (100 ug protein/ml) in a volume of 0.1 ml in combination with varying amounts of unlabelled Bt2 or Bt14. After a 30 min incubation period at 22° C the bound and free toxin were separated.

Figures 7 and 8 show the binding of ^{125}I-labeled toxins to P. brassicae brush border membrane vesicles. Vesicles were incubated with labeled toxin [in Fig. 7: ^{125}I-Bt2-toxin (1.05nM); in Fig. 8: ^{125}I-Bt14-toxin (1.4nM)] in the presence of increasing concentrate of Bt2 toxin (o) or Bt14 toxin (●). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample. Figure 7 shows the binding of labelled Bt2 P. brassicae BBMV, and Figure 8 shows the binding of labelled Bt14 P. brassicae BBMV.

The competition data demonstrate the presence of high affinity binding sites both for Bt2 and Bt14, as well as the almost complete absence of competition of Bt14 for the Bt2 binding sites and of Bt14 for the Bt2 binding sites. This demonstrates that Bt2 and Bt14 are non-competitively binding toxins. Hence they are useful to prevent the development of Pieris brassicae resistance against B. thuringiensis ICP's expressed in Brassica sp. Calculated Kd and Rt values were from these experiments were:

Bt2:	Kd = 2.8 nM, Rt = 12.9 pmol/mg vesicle protein
Bt14:	Kd = 8.4 nM, Rt = 21.4 pmol/mg vesicle protein

Binding of Bt2 and Bt15 to BBMV of M.sexta, M.brassicae, S.littoralis and P.interpunctella : an example of two non-competitively binding Lepidopteran ICPs

Bt2 and Bt15 toxins are both toxic to M.sexta (LC50's of 20 and 111 ng/cm2 respectively). They also show activity against M. brassicae, S. littoralis and P. interpunctella.

Labelled Bt2 (1.05 nM) or Bt15 (0.7 nM) were incubated with BBMV from M.sexta (100 ug protein/ ml) in a volume of 0.1 ml in combination with varying amounts of unlabelled Bt2 or Bt15. After a 30 min incubation period at 22° C the bound and free toxin were separated.

Figs. 9-10 show the binding of ^{125}I-labeled toxins to M. sexta brush border membrane vesicles. Vesicles were incubated with labeled toxin [in Fig. 9: ^{125}I-Bt2-toxin (1.05nM); in Fig. 10: ^{125}I-Bt15-toxin (0.7nM)] in the presence of increasing concentrations of Bt2-toxin (0) or Bt15-toxin (●). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample. Figure 9 shows the data for binding of labelled Bt2, and Figure 10 shows the binding of labelled Bt15.

The competition data demonstrate the presence of high affinity binding sites both for Bt2 and Bt15, as well as the complete absence of competition of Bt15 for the Bt2 binding sites and of Bt2 for the Bt15 binding sites. This demonstrates that Bt2 and Bt15 are non-competitively binding toxins. Hence the combination of Bt2 and Bt15 is useful to prevent the development of resistance of M.sexta against B. thuringiensis ICP's expressed in tobacco or other crops in which Manduca sp. are a pest. Calculated Kd and Rt values are:

Bt2:	Kd = 0.4 nM, Rt = 3.4 pmol/mg vesicle protein
Bt15:	Kd = 0.3 nM Kd2 = 2.9 nM, Rt1 = 5.9 and Rt2 = 6.7 pmol/mg vesicle protein (2 distinct high affinity receptor sites are present).

Similar studies were performed for M. brassicae, S. littoralis and P. interpunctella. Although LD50, Kd and Rt values differed substantially the essential observation that Bt2 and Bt15 are both toxic and are non-

competitively binding toxins was confirmed in these three insect species. Thus it is also a useful toxin combination to prevent resistance of M. brassicae against ICP's in Brassica sp. or to prevent resistance of Spodoptera species against ICP's expressed in any of the crop plants in which Spodoptera species are a pest.

Binding of Bt2 and Bt4 to BBMV of M. sexta: an example of two non-competitively binding Lepidopteran ICPs

Both Bt2 and Bt4 toxins are toxic to Manduca sexta. LD50 values are 20 and 10 ng.cm2 respectively. No mutual competition of Bt2 for binding of labelled Bt4 and of Bt4 for binding of labelled Bt2 was observed, demonstrating that Bt2 and BT4 are non-competitively binding toxins.

Binding of Bt15 and Bt18 to BBMV of S. littoralis: an example of two non-competitively binding Lepidopteran ICPs

Both Bt15 and Bt18 toxins are toxic to S. littoralis. LD 50 values are 93 and 88 ng toxin.cm2 respectively. Labelled Bt15 (0.7 nM) or Bt18 (0.9 nM) were incubated with 100 ug of vesicle protein from S. littoralis in combination with varying amounts of unlabelled Bt15 or Bt18 toxin. After a 45 min incubation period bound and free toxin were separated. Binding data demonstrate high affinity binding for both Bt15 and Bt18 to S. littoralis BBMV. As illustrated in figures 11 and 12, the entire population of receptor sites of Bt15 was not saturable with Bt18, neither was the entire population of receptor sites of Bt18 saturable with Bt15.

Binding of Bt13 and Bt22 to BBMV of L. decemlineata : an example of two non-competitively binding Coleopteran ICPs.

Both Bt13 and Bt22 toxins are toxic to L. decemlineata. LD 50 values are o.8 and 1.1 ug toxin/ml respectively. Labelled Bt13 (1 nM) or Bt22 (0.7 nM) were incubated with 100 ug of vesicle protein.ml from S. littoralis in combination with varying amounts of unlabelled Bt13 or Bt22 toxin. After a 45 min incubation period bound and free toxin were separated. Binding data demonstrate high affinity binding for both Bt13 and Bt22 to S. littoralis BBMV. The entire population of receptor sites of Bt13 was not saturable with Bt22. neither was the entire population of receptor sites of Bt22 saturable with Bt13.

Binding of Bt2 and Bt18 to BBMV of M. secta: an example of two non-competitively binding Lepidopteran ICPs.

Both Bt2 and Bt18 toxins are toxic to M. sexta, and LD 50 values are 20 to 73 ng toxin cm^2 respectively. Labelled Bt2 (1.05nM) or Bt18 (0.7nm) were incubated with 100 ug.ml of vesicle protein from M. sexta in combination with varying amounts of unlabelled Bt2 or Bt18 toxin. After a 45 min. incubation period, bound and free toxin were separated. Binding data (Figs. 11-12) demonstrate high affinity binding for both Bt2 and Bt18 to M. sexta BBMV. The entire population of receptor sites of Bt2 was not saturable with Bt18. neither was the entire population of receptor sites of Bt18 saturable with Bt2. Calculated Kd and Rt values are:

Bt2:	Kd = 0.4 nM, Rt = 3.4 pmol.mg vesicle protein.
Bt18:	Kd1 = 0.04 nM, Rt1 = 2.2 pmoles/mg vesicle protein and Kd2 = 168nM Rt2 = 194 pmoles/mg vesicle protein (2 distinct receptor sites for Bt18 are present).

A list of non-competitively binding anti-Lepidopteran ICP combinations and anti-Coleopteran ICP combinations is given below, together with their common target insect species in which non-competitivity has been demonstrated

Bt2-Bt15 (Manduca sexta, Sopdoptera littoralis, Pieris brassicae, Mamestra brassicae, Plodia interpunctella)
Bt2-Bt18 (Manduca sexta, Spodoptera littoralis)

Bt2-Bt14 (Pieris brassicae)
Bt2-Bt4 (Manduca sexta)
Bt15-Bt18 (Manduca sexta, Spodoptera littoralis)
Bt14-Bt15 (Pieris brassicae)
Bt15-Bt4 (Manduca sexta)
Bt18-Bt4 (Manduca sexta)
Bt18-Bt14 (Pieris brassicae)
Bt18-Bt4 (Manduca sexta)
Bt13-Bt21 (Leptinotarsa decemlineata)
Bt13-Bt22 (Leptinotarsa decemlineata)
Bt21-Bt22 (Leptinotarsa decemlineata)

This list is not believed to be exhaustive, and it will be clear that the competitivity of novel toxin combinations, even of toxins which are still to be discovered, can be investigated using a similar approach for any given insect species.

The above list of toxin combinations for a given insect pests will immediately suggest to those skilled in the art a number of host plants in which the expression of these combinations is useful : e.g. the combination of Bt2-Bt14 in Brassica to prevent resistance of Pieris brassicae against the ICP's expressed in the plant.

EXAMPLE 7: Selection for resistance of Manduca sexta (tobacco hornworm)

A selection experiment consists in exposing a large number of larvae to a concentration of toxin in diet killing e.g. 50-90 % of the larvae. The surviving larvae are again exposed to toxin concentrations killing a similar proportion of the larvae and this process is continued for several generations. The sensitivity of the larvae to the toxin is investigated after each four generations of selection.

We performed selections for 20 generations with Bt2 alone, with Bt18 alone and with a 1/4 by weigth Bt2/Bt18 mixture.

LC50 values of our reference strain for Bt2, Bt18 and the 1/4 Bt2 Bt18 mixture respectively were the following : 20 ng/cm2, 73 ng/cm2 and 62 ng/cm2 of diet.

Selection was initiated at with concentrations killing around 75 % of the animals. After 4 generations of selection survival increased in both the Bt2 and the Bt18 selection to around 70 %, no such increase was obtained in the selection with the combination of Bt2/Bt18. Dosages were again increased to calculated LC75 values. This was repeated every 4 generations. The selection process was thus continued to the 20th generation.

Final results were the following (LC50 of the 20th generation)

- Bt2 selection:	LC50 Bt2 6400 ug/g (320 times decreased sensitivity)
- Bt18 selection:	LC50 Bt15 15100 ug/g (207 times decreased sensitivity)
- Bt2/Bt18 selection:	LC50 Bt2/Bt15 181 ug/g (3 times decreased sensitivity).

Thus the decrease in sensitivity was about 100 times slower in the combined selection experiment.

Receptor binding in the three selected strains was investigated with Bt2 and Bt18 and compared to those of the reference strain (non-selected strain).

Binding characteristics reference strain :

Bt2:	Kd = 0.4 nM, Rt = 3.4 pmol/mg vesicle protein
Bt18:	Kd1 = 0.04 nM, Rt1 = 2.2 pmoles/mg vesicle protein and Kd2 = 168nM, Rt2 = 194 pmoles/mg vesicle protein (2 distinct receptor sites for Bt18 are present).

Figures 11 and 12 show the binding of ^{125}I-labeled toxins to M. sexta brush border membrane vesicle. Vesicles were incubated with labeled toxin [in Fig. 11: ^{125}I-Bt2-toxin (1.05nM); in Fig. 12: ^{125}I-Bt18-toxin (0.7nm)] in the presence of increasing concentrations of Bt2-toxin (0), or Bt18-toxin (●). Binding is

expressed as percentage of the amount bound upon incubation with labeled toxin alone. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample.

The Bt2 selected strain showed no detectable high affinity binding of Bt2 whereas its Bt18 binding characteristics remained close to the reference strain. (Bt18: Kd1 = 0.03 nM, Rt1 = 2.8 pmoles/mg vesicle protein and Kd2 = 199nM, Rt2 = 109 pmoles/mg vesicle protein -2 distinct receptor sites for Bt18 are still present).

The Bt18 selected strain lost the high affinity receptor site for Bt18. The lower affinity site for Bt18 was still present in lower concentration than in the reference strain (Kd = 189 nM, Rt = 43 nM). Bt2 binding site concentration increased markedly compared to the reference strain (Kd = 0.4 nM, Rt = 20.8 pmoles/mg vesicle protein. This strain had a Bt2 sensitivity of $LC_{50} = 4$ ng/cm^2. Thus, its sensitivity for Bt2 had increased as compared to the reference strain ($LC_{50} = 20$ ng/cm^2).

The Bt2 Bt18 selected strain showed a slight but statistically non-significant decrease in Bt18 binding site concentration. (Bt2: Kd = 0.4 nM, Rt = 3.4 pmol/mg vesicle protein ; Bt18 : Kd1 = 0.04 nM, Rt1 = 1.0 pmoles/mg vesicle protein and Kd2 = 168nM, Rt2 = 194 pmoles/mg vesicle protein (2 distinct receptor sites for Bt18 are present) .

These data demonstrate that in the two selection lines where resistance occurred, the mechanism was situated at the receptor level. Changes in receptor site are shown to be the most likely mechanism of resistance to B. thuringiensis ICPs.

EXAMPLE 8: Separate transfer of two ICP genes comprised within individual transcriptional units to the genome of plant cells

Two procedures are described for the combined expression of bt2 and bt15 in transgenic tomato plants. These procedures are based on the transfer of two chimeric ICP genes not linked within the same DNA fragment to the genome of the plant of interest.

A first procedure is based on sequential transformation steps in which a plant already transformed with a first chimeric ICP gene is retransformed in order to introduce a second ICP gene. The sequential transformation makes use of two different selectable marker genes, being the resistance genes for kanamycin and phosphinotricin acetyl transferase (PPT) which confers resistance to phoshinotricin (use of both selectable markers is already described in De Block et al., 1987). The second procedure is based on the cotransformation of two chimeric ICP genes localized on different plasmids in a single step. The integration of both ICP genes can be selected by making use of the two selectable markers conferring resistance to Km and PPT linked with the respective ICP genes. In this example, we describe Ti-plasmid vectors for Agrobacterium mediated transformation of these chimeric ICP genes.

In EP publication 0193259, pGSH163 has been described. This plasmid contains the following chimeric genes between the T-DNA border repeats : a gene fragment encoding the toxin part of bt2 gene under control of the TR2′ promoter and the neo gene under control of the TR1′ promoter. The 3′ ends of the T-DNA gene 7 and octopine synthase respectively provide information for the 3′ end formation of transcripts.

A chimeric bt15 gene containing a gene fragment encoding the toxin part of bt15 under the control of the TR2′ promoter was constructed in the following way. pOH50 consists of pUC18 with the whole bt15 gene under control of the lac promoter. A HindIII-BglII fragment was cloned in pMa5-8 yielding pJB3. By site directed mutagenesis as described, a NcoI site was created at the initiation codon to yield pVE29. A fragment containing the truncated gene fragment of the bt15 gene with a translational stop codon was obtained by isolation of BclI-ClaI from pOH50 and cloning in pLK91 yielding pHW38. The whole toxin gene fragment was reconstructed under control of the tac promoter yielding pVE35 by ligation of a ClaI-PstI fragment from pHW38, a NcoI-ClaI fragment from pVE29 and a NcoI-PstI fragment from pOH48. A truncated bt15 gene fragment with a NcoI site at the initiation codon was obtained from pVE35 as a 1980 NcoI-BamHI fragment and cloned in pGSJ141, digested with ClaI and BamHI. pGSJ141 has been described in EP application n° 88402115.5. Ligation of the filled ClaI site to the filled NcoI site yielded a chimeric TR2′-bt15 truncated - 3′g7 construct (pTVE47). As a selectable marker in this plasmid, the bar gene encoding phosphinothricin acetyl transferase and conferring resistance to PPT was used. A chimeric bar gene containing the bar gene under control of the 35S promoter and followed by the 3′ end of the octopine synthase was introduced in pTVE47. From pDE110, a 35S-bar-3′ocs fragment was obtained as a StuI-HindIII fragment and was cloned in pTVE47 digested with PstI and HindIII. This yielded the plasmid pTHW88 which contains the truncated bt15 gene under control of the TR2′ promoter and the bar gene under control of the 35S promoter comprised between the T-DNA border repeats. Plasmid pGSH163 is

cointegration type Ti-plasmid vector, whereas pTHW88 is a binary type Ti-plasmid vector as described in EP publication 0193259.

Both plasmids were mobilized in the A. tumefaciens strain C58C1Rif (pGV2260) according to Deblaere et al., (1988). In the sequential transformation procedure, tomato has been transformed according to De Block et al., 1987 with the A. tumefaciens strain C58C1Rif carrying pGS1163 resulting from the cointegration of pGSH163 and pGV2260. Individual transformants were selected for kanamycin resistance, and regenerated plants were characterized for expression of the truncated bt2 gene according to Vaeck et al., (1987). One representative transformant was subsequently retransformed with the A. tumefaciens strain C58C1Rif (pGV2260) (pTHW88), and transformants were selected for PPT resistance. Using the cotransformation procedure, the respective Agrobacteria strains carrying the cointegrate vector pGS1163 and the binary vector pTHW88 were used for transformation of tomato. Individual plants were selected for resistance to Km and PPT.

Schematically shown in Fig. 15 are:

a) construction of pVE29:	bt15 N-terminal gene fragment with NcoI site introduced at ATG initiation codon.
b) construction of pVE35:	bt15 C-terminal truncated gene fragment under control of the tac promoter.
c) construction of pTHW88:	binary T-DNA vector with a chimeric bt15 gene and a chimeric bar gene within the T-DNA border repeats.

In both cases, co-expression of the two ICP genes in the individual transformants was evaluated by insect toxicity tests as described in EP publication 0193259 and by biochemical means. Specific RNA probes allow to quantitate the transcript levels, monoclonal antibodies cross-reacting with the respective gene products allow to quantitate the respective gene products in ELISA tests and specific DNA probes allow to characterize the genome integrations.

These procedures also could be applied when one or both ICP genes are part of a hybrid gene. For example, the same strategy as described could be followed with the plasmid vectors pGSH152, containing a chimeric truncated-neo bt2 hybrid gene under control of the TR2′ promoter and pTHW88 in suitable Agrobacterium strains.

EXAMPLE 9: Transfer of two chimeric ICP genes linked within the same DNA to the genome of plant cells

The strategy used is based on the organization of two independent chimeric ICP genes between the T-DNA border repeats of a single vector. Binding studies indicated that the Bt2 and Bt14 toxins are two non-competitively binding ICPs with insecticidal activity towards Pieris brassicae. For expression in plants, both the bt2 and bt14 genes can be co-expressed to prevent insect resistance development. For the design of a plasmid vector with each ICP gene under the control of a separate promoter, several possibilities can be envisaged: 1) three chimeric constructs carrying the truncated bt2 and bt14 genes and a selectable marker, respectively, or 2) a hybrid of a truncated gene fragment (bt2 orbt14) and the neo gene can be used in combination with a truncated bt14 or bt2 gene. This example describes the construction of the vector pTHW94 for plant transformation carrying the following chimeric ICP genes between the T-DNA border repeats: a truncated bt2 gene fragment under the control of the TR2′ promoter and a hybrid truncated bt14-neo gene under the control of the TR1′ promoter. The 3′ end of the T-DNA gene 7 and octopine synthase respectively provide information for proper 3′ end formation. pTHW94 is being deposited at Deutsche Sammlung von Mikroorganismen und Zellculturen ("DSM"), Braunschweig, Federal Republic of Germany.

Schematically shown in Fig. 16 are:

a) construction of pHW44:	bt14 N-terminal gene fragment with NcoI site introduced at ATG initiation codon.
b) construction of pHW67:	reconstruction of the bt14 gene under control of the tac promoter.
c) construciton of pHW71:	construction of a hybrid bt14 ox-neo gene under control of the tac promoter.
d) construction of pTHW94:	binary T-DNA vector with a chimeric bt14 gene and a chimeric bt2 gene within the T-DNA border repeats.

The pTHW94 vector is a binary vector derived from pGSC1701A2 and is mobilized into the Agrobacterium strain C58C1Rif (pMP90). This strain was used to transform Brassica napus according to the procedure described by De Block et al. (in preparation). Transformants are selected on Km, and regenerated plants are analyzed for expression of both ICP gene products by insect toxicity tests and biochemical tests.

EXAMPLE 10: Expression of two ICP genes in a hybrid construct

In order to obtain a combined and simultaneous expression of two ICP genes, truncated gene fragments encoding the toxic parts of two different ICPs can be fused in a proper reading frame and placed as a hybrid gene under the control of the same promoter in a chimaeric gene construct. Toxic cores from certain ICPs can be liberated from their protoxins by protease activation at the N- and/or C- terminal end. Thus, hybrid genes can be designed with one or more regions encoding protease cleavage site(s) at the fusion point(s) of two or more ICP genes.

The simultaneous co-expression of bt2 and bt14 genes can be obtained by constructing a hybrid gene composed of a truncated bt14 gene fragment fused to a truncated bt2 gene fragment. Schematically shown in Figure 17 is the construction of such a hybrid bt2-bt14 gene with a C-terminal bt2 gene fragment (bt860) encoding the toxic core of the Bt2 protoxin in frame with a C-terminal truncated bt14 gene fragment encoding the toxic core of the Bt14 protoxin. In practice, the BclI site in the bt2 gene localized downstream of the trypsin cleavage site can be fused in frame with the NcoI site introduced at the N-terminal end of the truncated bt14 gene fragment. To this end, the plasmids pLBKm860 (EP publication 0193259) and pHW67 are used. pLBKm860 contains a hybrid bt2-neo gene under control of the lambda P_L promoter. The bt2 gene moiety in the hybrid gene is a C-terminal truncated bt2 gene fragment, indicated as bt860 (in Fig. 17) (see also Vaeck et al. 1987). The construction of pHW67 is described in Fig. 16. pHW67 contains a C-terminal truncated bt14 gene fragment (bt14tox) with a NcoI site at the ATG initiation codon, a translation stop codon located at the BclI site of the intact bt14 gene and a BamHI site downstream of the whole gene fragment. To fuse both gene fragments in the proper reading frame, the BclI and NcoI ends of the respective plasmids are treated with Klenow DNA polymerase and S1 nuclease as indicated in Figure 16. The resulting plasmid pJB100 contains the hybrid bt860-bt14tox gene under control of the lambda P_L promoter and directs the expression in E. coli of a fusion protein with the expected mobility on SDS-PAGE.

Crude extracts of the E. coli strain show the toxicity of the fusion protein, expressed by the strain, against P. brassicae. It has also been confirmed by N-terminal amino acid sequence analyses of the fusion protein produced by the E. coli strain that the N-terminal amino acids from the Bt14 protoxin are processed upon activation. The bt2-bt14 hybrid gene product has thus two potential protease cleavage sites. Subsequently, this hybrid gene can be inserted into a vector for plant transformation and placed under control of a suitable promoter and transferred to the genome of a plant of interest (EP publication 0,193,259).

Table 1

Gene	Bt strain	Host range	amino acids encoded	predicted MW(kDa) of encoded aminoacids	Disclosure of nucleotide sequence
bt3	HD-1 kurstaki	L	1176	133.2	Schnepf et al.,1985
bt2	berliner 1715	L	1155	131	Höfte et al.,1986
bt73	HD-73	L	1178	133.3	Adang et al, 1985
bt14	HD-68 aizawai	L	1207	138	Brizzard and Whitely, 1988
bt15	entomocidus HD-110	L	1189	134.8	Fig. 14
bt4	entomocidus HD-110	L	1165	132.5	Fig. 13
bt18	darmstadiensis HD-146	L	1171	133	EP appln. 88402241.9
bt13	BtS1,DSM4288 22/10/87	C	644	73.1	EP appln. 88402115.5
bt21	BtPGSI208, DSM 5131, 19/1/89	C	651	74.2	EP appln. 89400428.2

Table 1 (continued)

Gene	Bt strain	Host range	amino acids encoded	predicted MW(kDa) of encoded aminoacids	Disclosure of nucleotide sequence
bt22	BtPGSI245, DSM 5132, 19/1/89	C	1138	129	EP appln. 8940028.2
P2	HD-263	L/D	633	70.9	Donovan et al, 1988
Cry B2	HD-1	L	633	70.8	Widner and Whiteley, 1989

REFERENCES

- Adang M., Staver M., Rocheleau T., Leighton J., Barker R. and Thompson D. (1985), Gene 36, 289-300.
- Barton K., Whiteley H. and Yang N.-S. (1987), Plant Physiol. 85, 1103-1109.
- Bernard H., Remaut E., Hersfield M., Das H., Helinski D., Yanofski C. and Franklin N. (1979), Gene 5, 59-76.
- Bell R. and Joachim F. (1976), Ann. Entomol. Soc. Am. 69, 365-373.
- Botterman J. and Zabeau M. (1987), DNA 6, 583-591.
- Bradford M. (1976), Anal. Biochem. 72, 248-254.
- Brattsten L., Holyoke C., Leeper J. and Raffa K. (1986), Science 231, 1255-1260.
- Brizzard B. and Whiteley H. (1988), Nucleic Acids Research 16, 4168-4169.
- Deblaere R., Reynaerts A., Höfte H., Hernalsteens J-P, Leemans J. and Van Montagu M. (1988), Methods in Enzymol. 153, 277-292.
- De Block M., Botterman J., Vandewiele M., Dockx J., Thoen, Gosselé V., Rao Movva, Thompson C., Van Montagu M. and Leemans J. (1987), EMBO J. 6, 2513-2518.
- De Boer H., Comstock L. and Vasser M. (1983), Proc. Natl. Acad. Sci. USA 80, 21-25.
- de Framond A., Back E., Chilton W., Kayes L. and Chilton M-D (1986), Mol. Gen. Genet. 202, 125-131.
- De La Pena and Schell (1986), Nature 325, 274-276.
- Delauney A., Tabaeizadeh Z. and Verma D. (1988), Proc. Natl. Acad. Sci. USA 85, 4300-4304.
- Depicker A., Herman L., Jacobs A.,Schell J. and Van Montagu M. (1985), Mol. Gen. Genet. 201, 477-484.
- Donovan W., Dankoscik C. and Gilbert W. (1988), J. Bacteriol. 170, 4732-4738.
- Dulmage H.T and cooperators (1981), In : Microbial control of pests and plant diseases 1970-1980 (Ed. H.D. Burges), Academic Press. 193-222.
- Finney D. (1962), Probit Analysis (University Press, Cambridge), pp. 50-80.
- Fischhoff D., Bowdish K., Perlak F., Marrone P., McCormick S., Niedermeyer J., Dean D., Kuzano-Kretzmer K., Mayer E., Rochester D., Rogers S. and Fraley R. (1987), Bio/Technology 5, 807-812.
- Franck, Guilley, Jonard, Richards and Hirth (1980), Cell 21, 285-294.
- French B., Maul H. and Maul G. (1986), Anal. Biochem. 156, 417-423.
- Fuller F. (1982), Gene 19, 43-54.
- Gardner, Howarth, Hahn, Brown-Luedi, Shepard and Messing, Nucl. Acids Res. 9, 2871-2887.
- Goldberg L. and Margalit J. (1977), Mosq. News 37, 355-358.

- Goldman I., Arnold J. and Carlton B. (1986), J. Invert. Pathol. 47, 317-324.
- Gould F. (1988), Bioscience 38, 26-33.
- Haider M., Knowles B. and Ellar D. (1986), Eur. J. Biochem. 156, 531-540.
- Hofmann C., Lüthy P., Hütter R. and Pliska V. (1988a), Eur. J. Biochem. 173, 85-91.
- Hofmann C., Vanderbruggen H., Höfte H., Van Rie J., Jansens S. and Van Mellaert H. (1988b), Proc. Natl. Acad. Sci. USA 85, 7844-7848.
- Höfte H., Van Rie J., Jansens S., Van Houtven A., Verbruggen H. and Vaeck M. (1988), Appl. Environ. Microbiol. 54, 2010-2017.
- Höfte H., De Greve H., Seurinck J., Jansens S., Mahillon J., Ampe C., Vanderkerkhove J., Vanderbruggen H., Van Montagu M., Zabeau M. and Vaeck M. (1987), Eur. J. Biochem. 161, 273-280.
- Hsiung H. and Becker G. (1988), Biotech. and Genetic Engin. Rev. 6, 43-65.
- Hull and Howell (1987), Virology 86, 482-493.
- Hunter W. and Greenwood F. (1962), Nature 194, 495-496.
- Kozak M. (1987), Mol. Cell. Biol. 7, 3438-3445.
- Krebbers E., Herdies L., De Clercq A., Seurinck J., Leemans J., Van Damme J., Segura M.,Gheysen G., Van Montagu M. and Vandekerckhove J. (1988), Plant Physiol. 87, 859-866.
- Knowles B. and Ellar D. (1986), J. Cell. Sci 83, 89-101.
- Krieg A., Huger A., Langenbruch G. and Schnetter W. (1983), Z. Ang. Ent. 96, 500-508.
- Krieg A. and Langenbruch G. (1981), In : Microbial control of pests and plant diseases 1970-1980 (Ed. H.D. Burges), Academic Press, 837-986.
- Kirsch K. and Schmutterer H. (1988), J. Appl. Ent. 105, 249-255.
- Kronstad J., Schnepf H. and Whiteley H. (1983), J. Bacteriol. 154, 419-428.
- Mahillon J. and Delcour J. (1984), J. Microbiol. Methods 3, 69-73.
- Maxam A. and Gilbert W. (1980), Methods in Enzymol. 65, 499-560.
- McGaughey W. (1985), Science 229, 193-195.
- McGaughey W. and Beeman R. (1988), J. Econ. Entomol. 81, 28-33.
- Munson P. and Rodbard D. (1980), Anal. Biochem. 107, 220-239.
- Pazkowski and cooperators (1984), EMBO J 3, 2717-2722.
- Peleman J., Boerjan W., Engler G., Seurinck J., Botterman J., Alliote T., Van Montagu M. and Inzé D. (1989), The Plant Cell 1, 81-93.
- Remaut E., Stanssen P. and Fiers W. (1981), Gene 15, 81-93.
- Sandler S., Stayton M., Townsend J., Ralstan M., Bedbrook J. and Dunsmuir P. (1988), Plant Mol. Biol. 11, 301-310.
- Scatchard G. (1949), Ann. N.Y. Acad. Sci. 51, 660-672.
- Schocher R., Shillito R., Saul M.,Pazkowski J. and Potrykus I. (1986) Biotechnology 4, 1093-1096.
- Shields (1987), Nature 328, 12-13.
- Schnepf H., Wong H. and Whiteley H. (1985), J. Biol. Chem. 260, 6264-6272.
- Stanssens P., Remaut E. and Fiers W. (1985), Gene 36, 211-223.
- Stanssens P., McKeown Y., Friedrich K. and Fritz H. (1987) : "Oligo-nucleotide directed construction of mutations by the gapped duplex DNA method using the pMa/c plasmid vectors", published in the Collection of Experimental Procedures distributed at the EMBO course entitled "Directed mutagenesis and protein engineering" in July 1987 at the Max Planck Institut für Biochemie, Martinsried, FRG.
- Stone T., Sims S. and Marrone P. (1989), J. Invert. Pathol. 53, 228-234.
- Vaeck M., Reynaerts A., Höfte H., Jansens S., De Beukeleer M., Dean C. Zabeau M., Van Montagu M. and Leemans J. (1987), Nature 327, 33-37.
- Voller, Bidwell and Barlett (1976), In : Manual of Clinical Immunology (Eds. Rose and Friedman), pp. 506-512, American Society of Microbiology, Washington
- Velten J., Velten L., Hain R. and Schell J. (1984), EMBO J. 3, 2723-2730.
- Velten J. and Schell J. (1985), Nucl. Acids Res. 13, 6981-6998.
- Widner W. and Whiteley H. (1989), J. Bacteriol. 171, 965-974.
- Wolfersberger M., Lüthy P., Maurer A., Parenti P., Sacchi V., Giordana B. and Hanozet G. (1987), Comp. Biochem. Physiol. 86, 301-308.
- Yanisch-Perron C., Vieira J. and Messing J. (1985), Gene 33, 103-119.

Claims

1. A cell of a plant, characterized by: at least two B. thuringiensis genes stably inserted into the genome

of said plant; each of said genes encoding a different non-competitively binding insecticidal crystal protein for an insect species; whereby at least two different insecticidal crystal proteins can be produced by said cell which do not bind competitively to the brush border membrane of the columnar midgut epithelial cell of said insect species.

2. The cell of claim 1 wherein at least one marker gene, encoding a protein or polypeptide which renders said cell easily distinguishable from cells which do not contain said protein or polypeptide, is in the same genetic locus as at least one of said genes.

3. The cell of claim 1 or 2, wherein each of said genes is under the control of a separate promoter capable of directing gene expression in said cell and is provided with a separate signal for 3′ end formation and within a same transcriptional unit.

4. The cell of claim 2 or 3, in which said marker DNA is under the control of a separate promoter capable of directing gene expression in said plant cell and is provided with a signal for 3′ end formation within a transcriptional unit.

5. The cell of claim 1 or 2, wherein said genes are within a same transcriptional unit and under the control of a single promoter.

6. The cell of claim 5, wherein said marker gene is fused with at least one of said genes and is within said same transcriptional unit and under the control of said promoter.

7. The cell of claim 5 or 6, wherein a DNA fragment, encoding a protease sensitive amino acid sequence, is in said same transcriptional unit as said genes and intercalated in frame between said genes.

8. The cell of claim 5 or 6, wherein an intergenic DNA sequence allowing reinitiation of translation, is in said same transcriptional unit as said genes and intercalated between said genes.

9. The cell of anyone of claims 1 to 8, wherein two or three of said genes encode insecticidal proteins having activity against Lepidoptera species, particularly the following genes: bt2 and/or bt3 and/or bt73 and/or bt4 and/or bt14 and/or bt15 and/orbt18.

10. The cell of any of claims 1 to 8, wherein said genes encode insecticidal proteins having activity against a Coleoptera species, particularly the following genes: bt13, and/or bt21 and/or bt22.

11. The cell of any of claims 2 to 10 wherein said marker DNA is an herbicide resistance gene, particularly a sfr or sfrv gene, a gene encoding a modified target enzyme for a herbicide having lower affinity for the herbicide, particularly a modified 5-EPSP as target for glyphosate or a modified glutamine synthetase as target for a GS inhibitor, an antibiotic resistance gene, particularly NPTII.

12. The cell of any of claims 3 to 6, wherein said promoter is a constitutive promoter, particularly a 35S promoter, or a 35S3 promoter, a PNOS promoter, a POCS promoter, a wound inducible promoter, particularly a TR1′ or TR2′ promoter, a promoter which directs gene expression selectively in plant tissue having photosynthetic activity, particularly a SSU promoter, a tissue-specific promoter, particularly a tuber-specific promoter, or a stem-specific promoter or a seed-specific promoter.

13. A vector suitable for transforming a cell of a plant, particularly a plant capable of being infected with Agrobacterium, comprising said genes of any of claims 1 to 12.

14. A process for producing a plant having improved insect resistance and having said genes of any of claims 1 to 12 stably integrated into the nuclear genome of their cells, characterized by the non-biological steps of transforming a cell of said plant by introducing said genes into the nuclear genome of said cell and regenerating said plant and reproduction material from said cell.

15. A plant cell culture, containing the plant cell of any claims 1 to 12.

16. A plant, containing the plant cell of any of claims 1 to 12.

17. A plant, particularly brassica, tomato, tobacco, cotton or lettuce, containing the plant cell of any of claims 1 to 12, wherein said genes are particularly bt2 and bt15.

18. A plant, particularly brassica, tomato, tobacco or cotton or lettuce, containing the plant cell of any of claims 1 to 12, wherein said bt genes are particularly bt73 and bt15.

19. A plant, particularly tomato, tobacco, cotton, containing the plant cell of any of claims 1 to 12, wherein said genes are particularly bt2 and bt18.

20. A plant, particularly brassica, containing the plant cell of any of claims 1 to 12, wherein said genes are particularly bt2 and bt14.

21. A plant, particularly tomato or tobacco, containing the plant cell of any of claims 1 to 12, wherein said genes are particularly bt2 and bt4.

22. A plant, particularly tomato, cotton, containing the plant cell of any of claims 1 to 12, wherein said genes are particularly bt15 and bt18.

23. A plant, particularly brassica, containing the plant cell of any of claims 1 to 12, wherein said genes are particularly bt14 and bt15.

24. A plant, particularly potato, containing the plant cell of any of claims 1 to 12, wherein said genes are

particularly bt13 and bt21.

25. A plant, particularly potato, containing the plant cell of any of claims 1 to 12, wherein said genes are particularly bt21 and bt22.

26. A plant, particularly potato, containing the plant cell of any of claims 1 to 12, wherein said genes are particularly bt13 and bt22.

27. The cell of anyone of claims 1-12, made by a process as described hereinabove.

28. The plant of anyone of claims 15-26, made by a process as described hereinabove.

29. A method for rendering a plant resistant to an insect species by transforming the plant with said genes of any of claims 1-12.

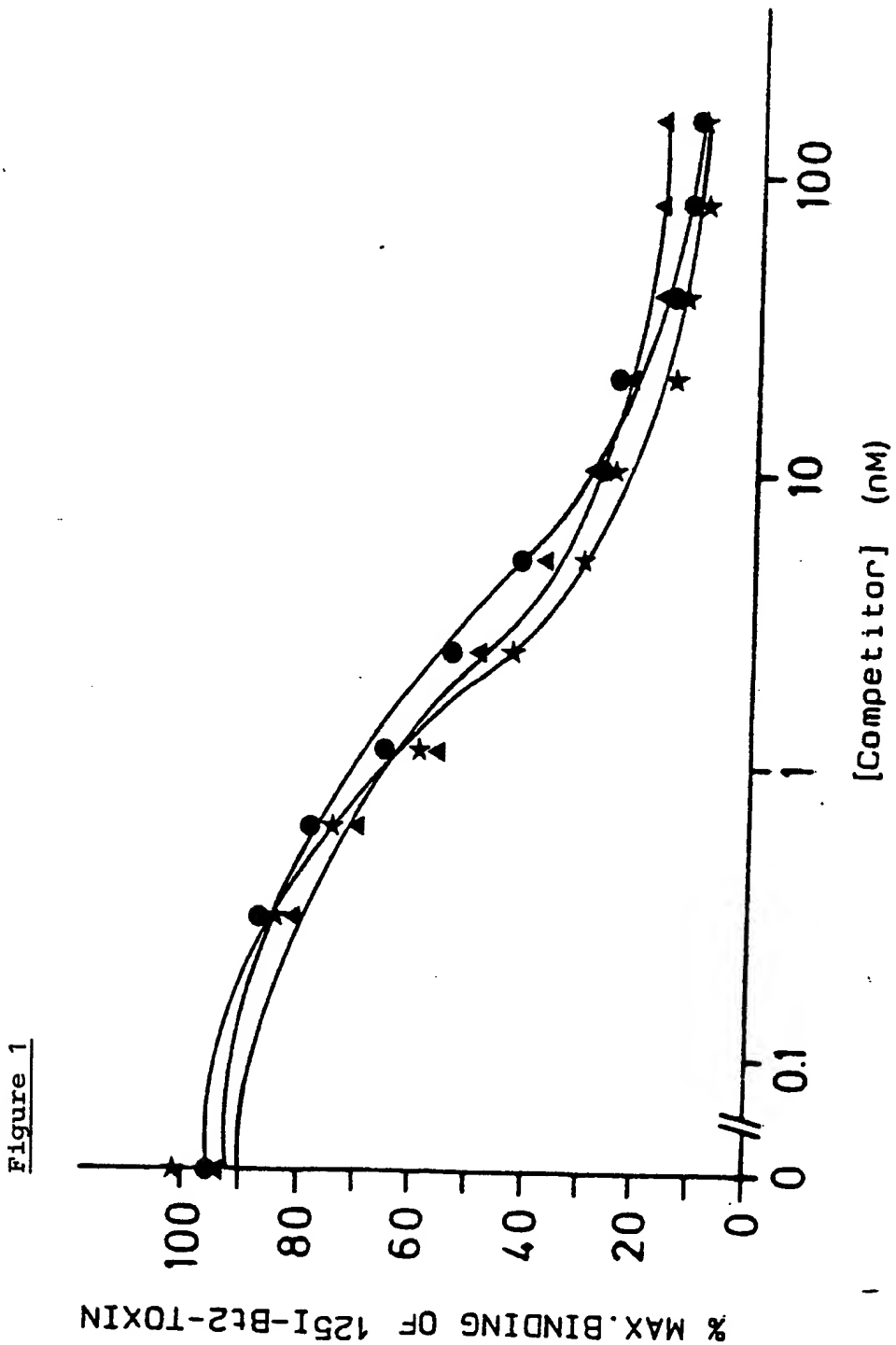

% MAX.BINDING OF 125I-Btz-TOXIN
100
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)

Figure 1

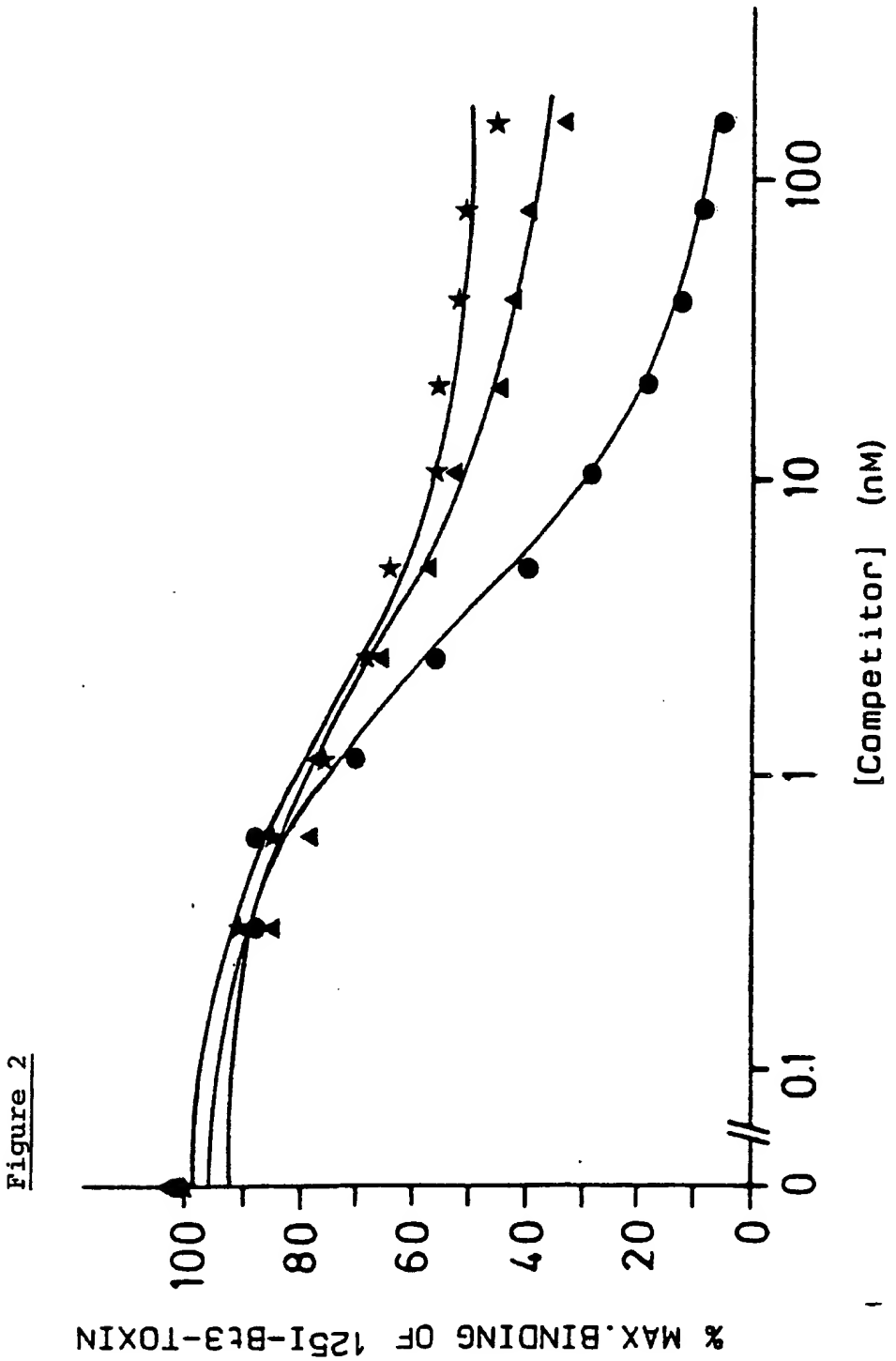

% MAX.BINDING OF 125I-Bt3-TOXIN
100
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)

Figure 2

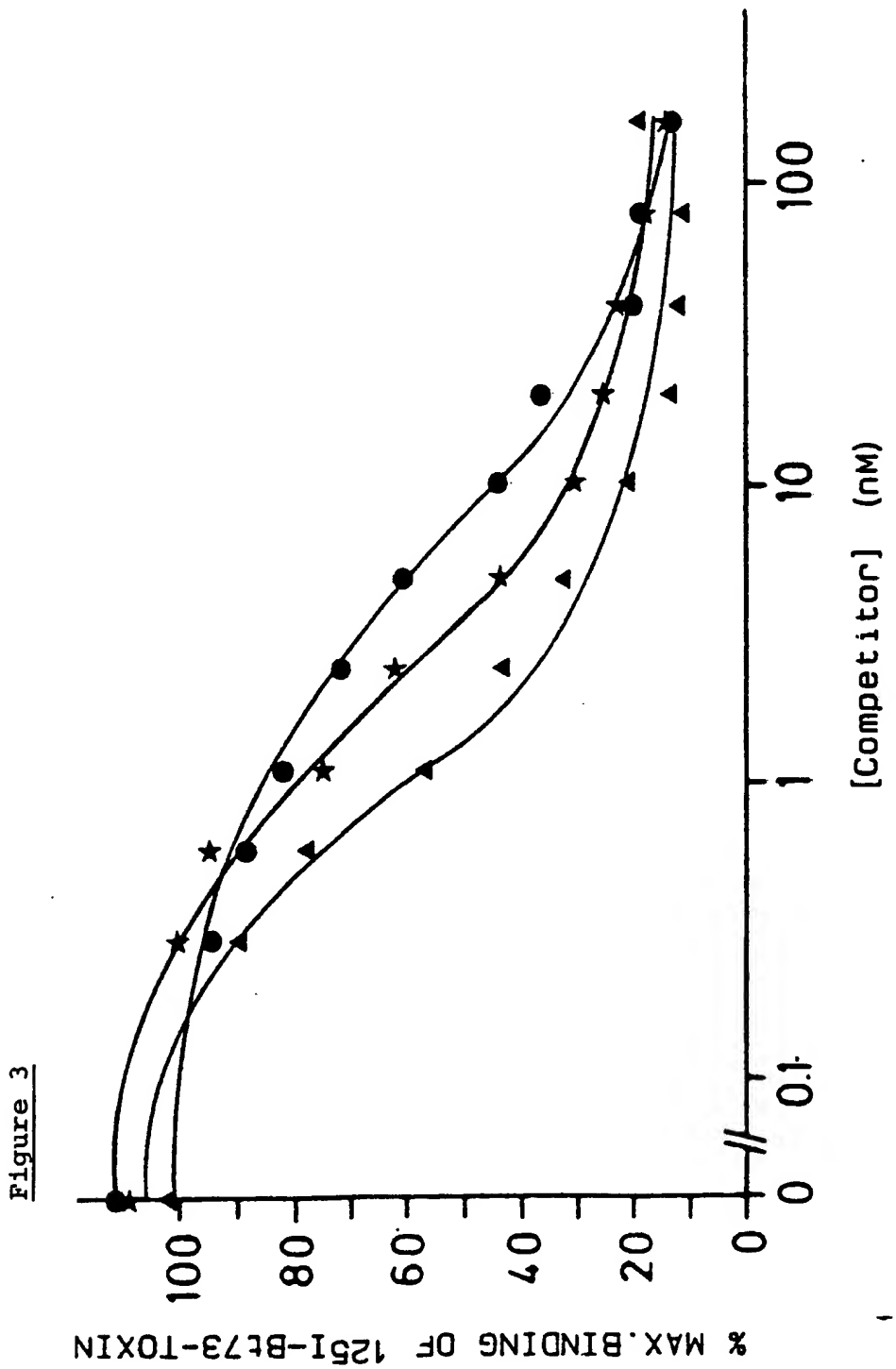

% MAX.BINDING OF 125I-Bt73-TOXIN
100
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)

Figure 3

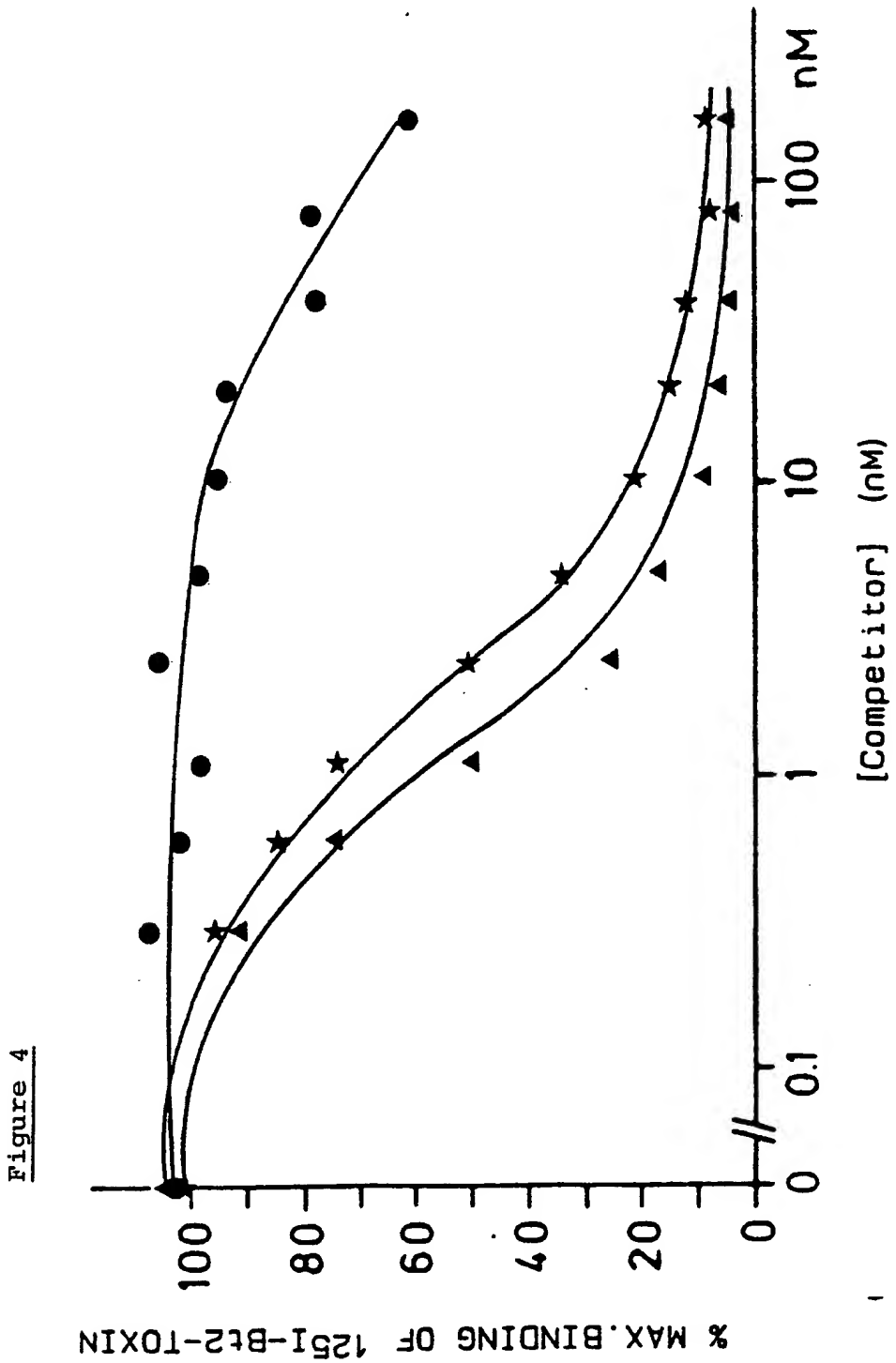

% MAX.BINDING OF 125I-Bt2-TOXIN
100
80
60
40
20
0
0
0.1
1
10
100 nM
[Competitor] (nM)

Figure 4

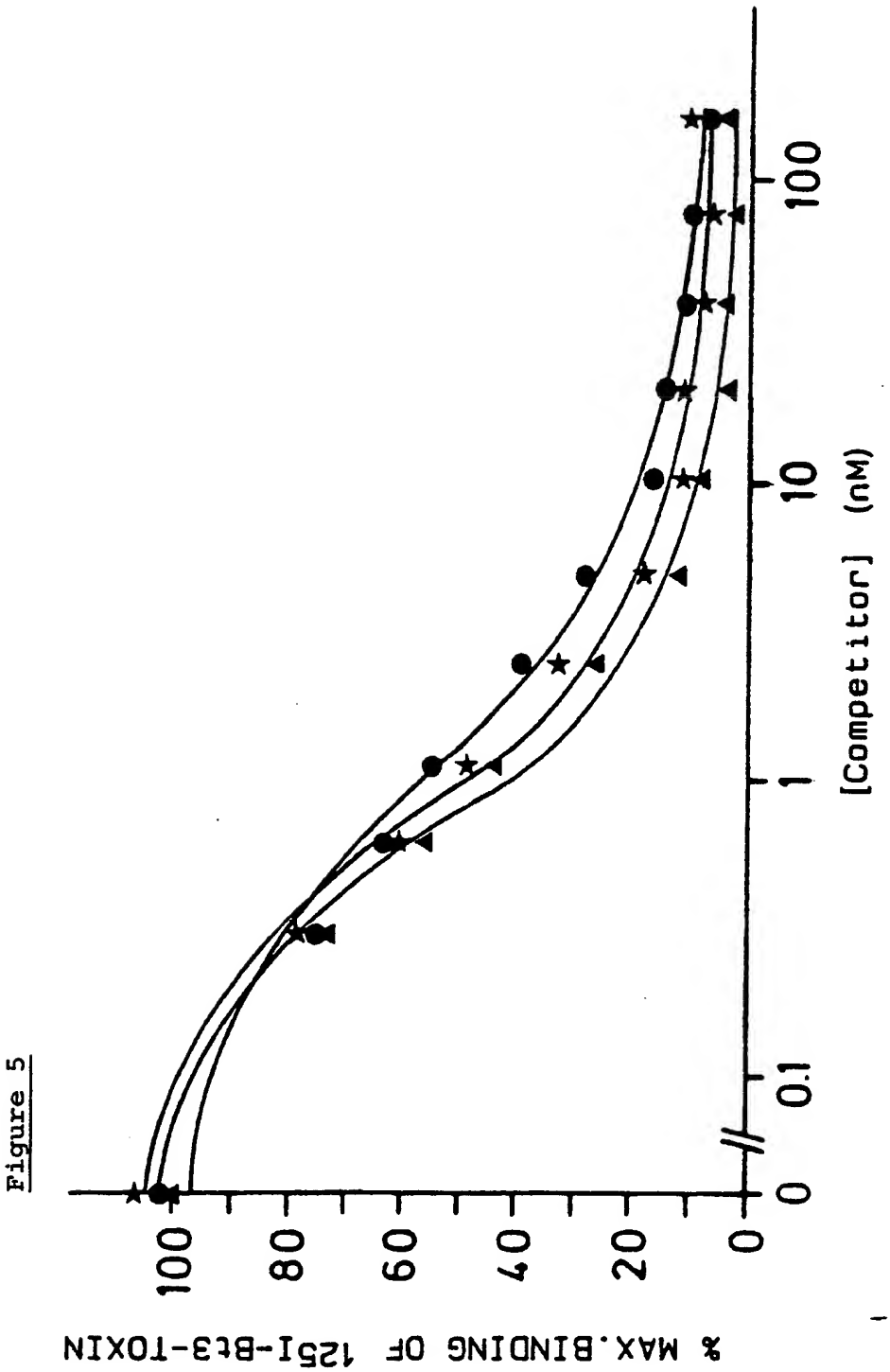

% MAX.BINDING OF 125I-Bt3-TOXIN
100
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)

Figure 5

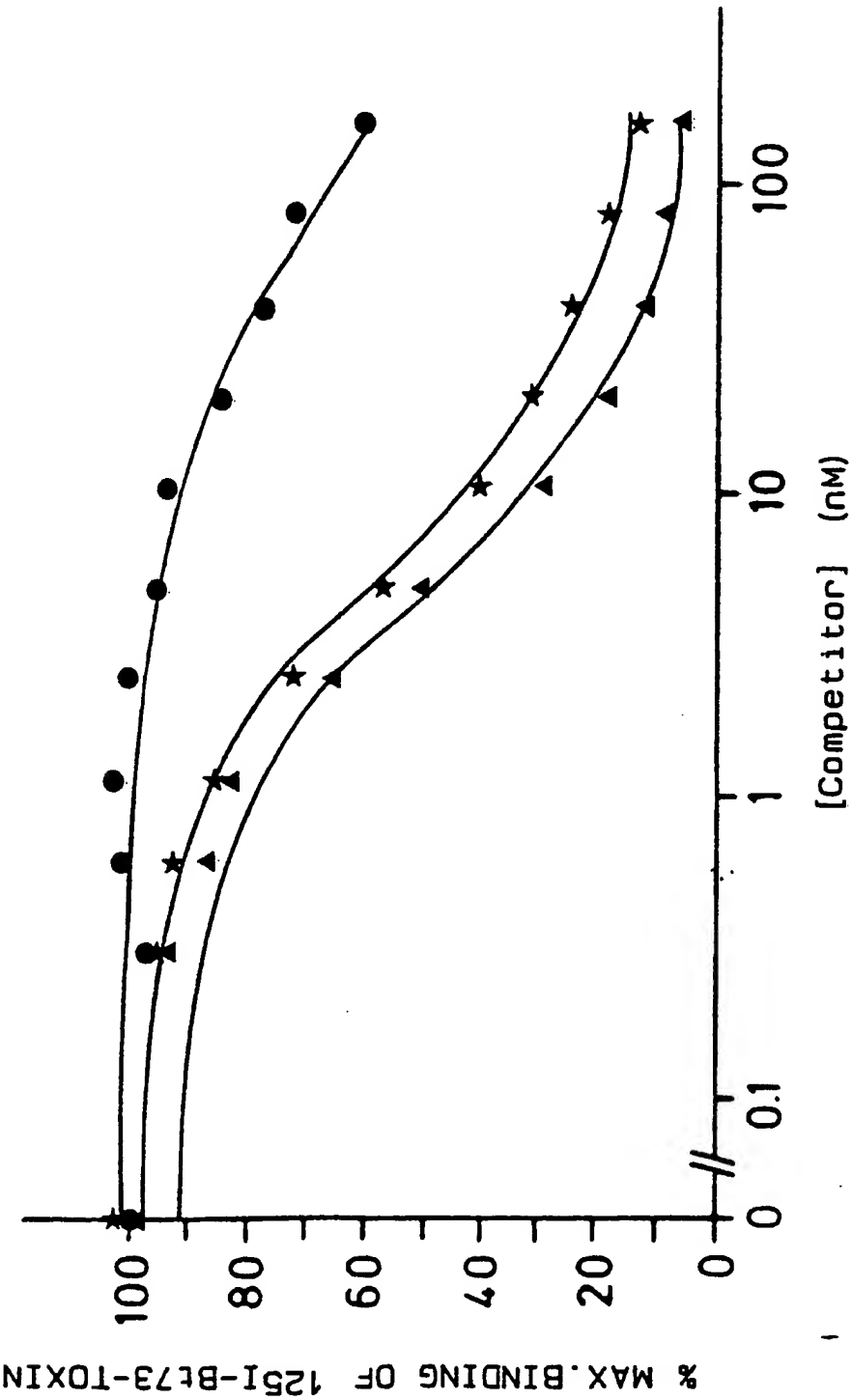

% MAX.BINDING OF 125I-Bt73-TOXIN
100
80
60
40
20
0
0
0.1
1
10
100
[Competitor] (nM)

Figure 6

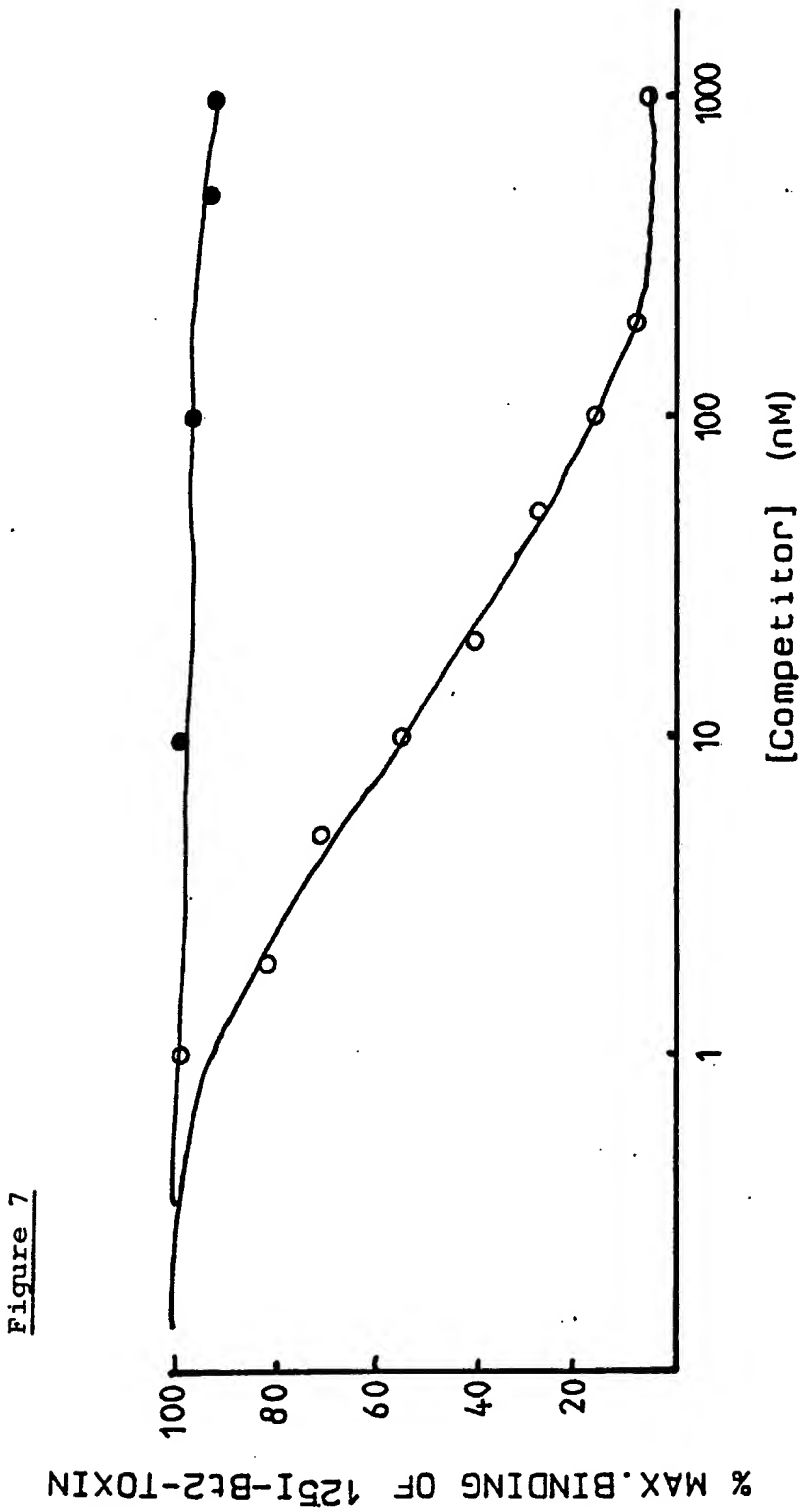

% MAX.BINDING OF 125I-Btx-TOXIN
100
80
60
40
20
1
10
100
1000
[Competitor] (nM)

Figure 7

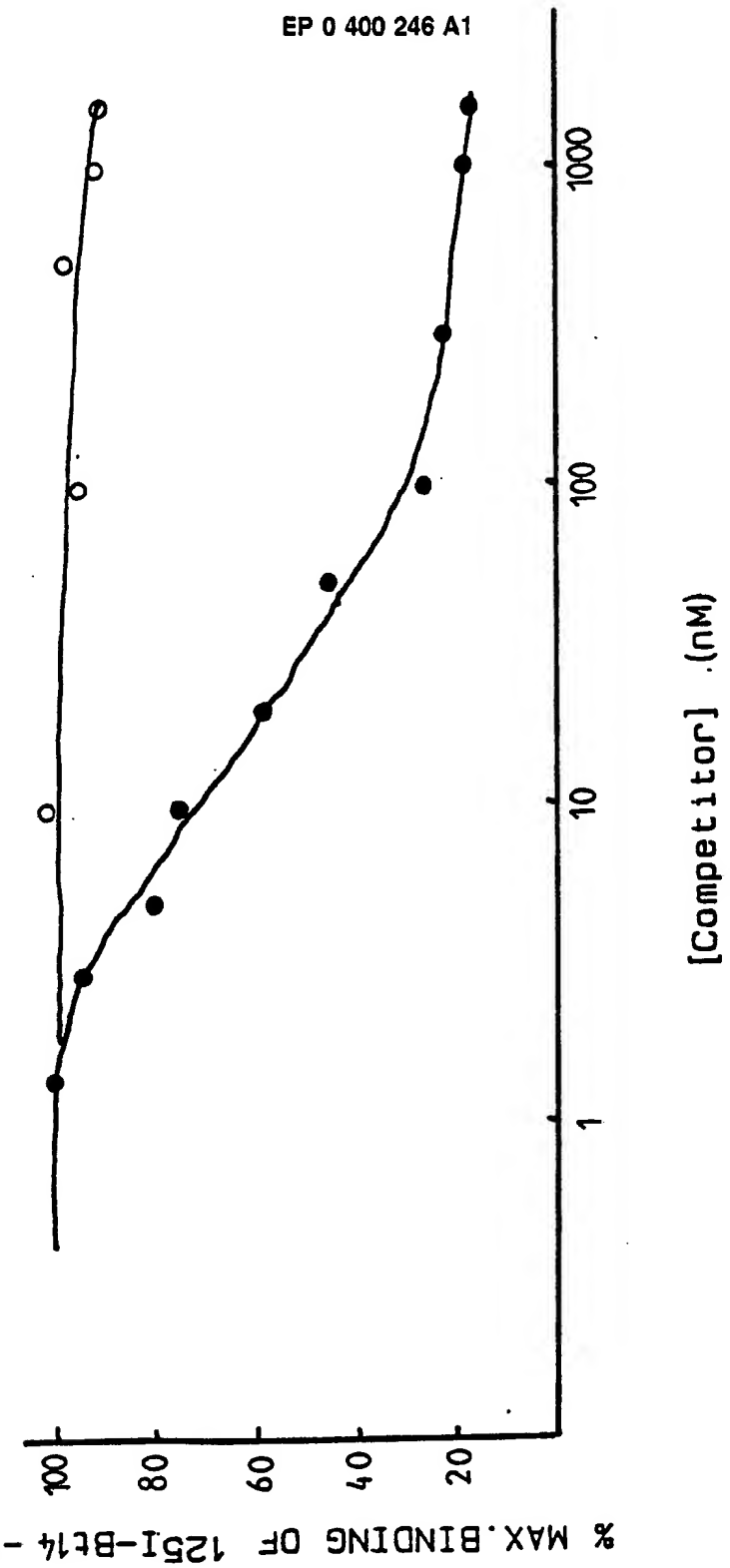

% MAX.BINDING OF 125I-Bt14 -TOXIN
100
80
60
40
20
1
10
100
1000
[Competitor] (nM)

Figure 8

Figure 9

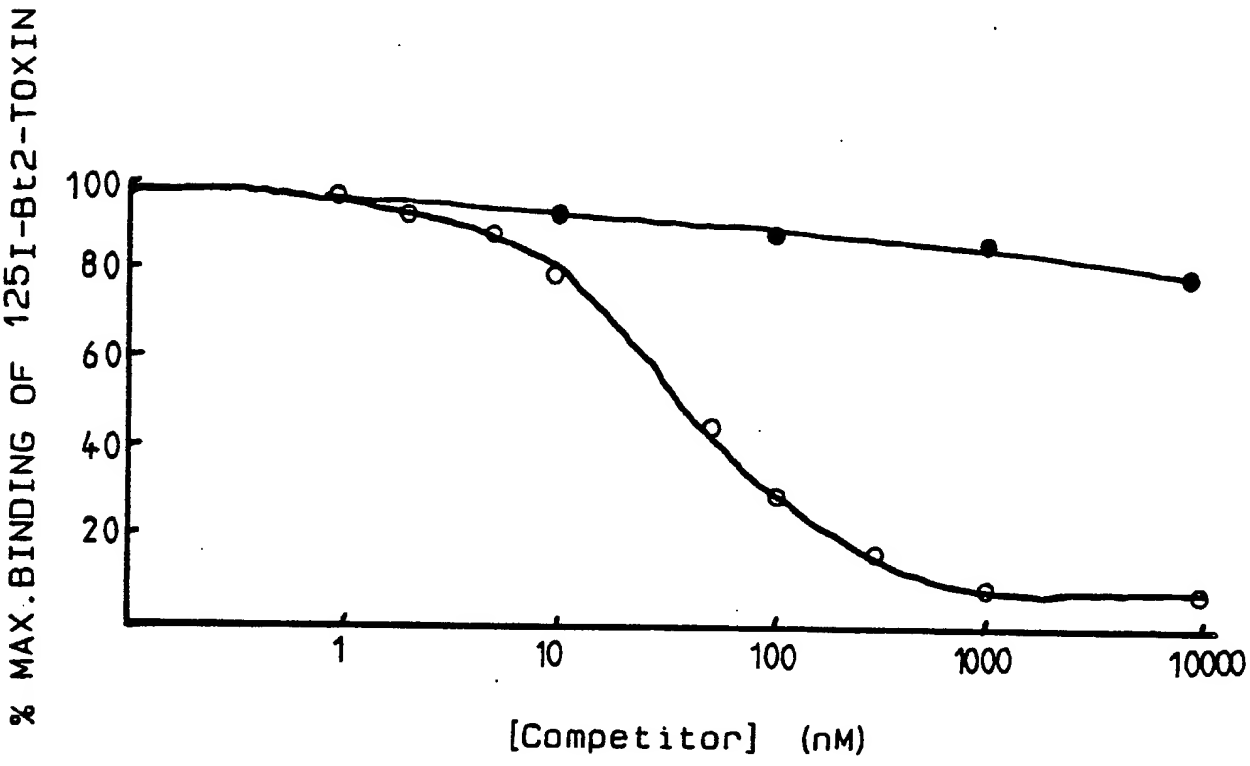

% MAX.BINDING OF 125I-Bt2-TOXIN
100
80
60
40
20
1
10
100
1000
10000
[Competitor] (nM)

Figure 10

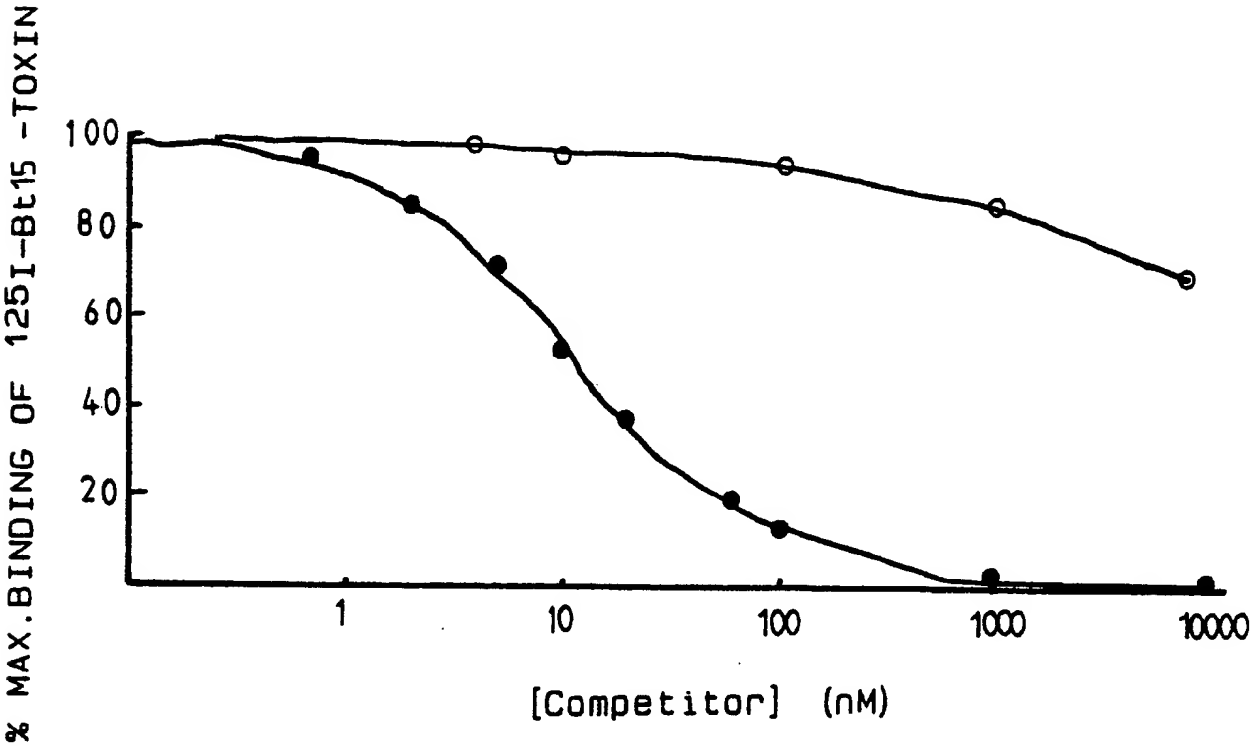

% MAX.BINDING OF 125I-Bt15 -TOXIN
100
80
60
40
20
1
10
100
1000
10000
[Competitor] (nM)

Figure 11

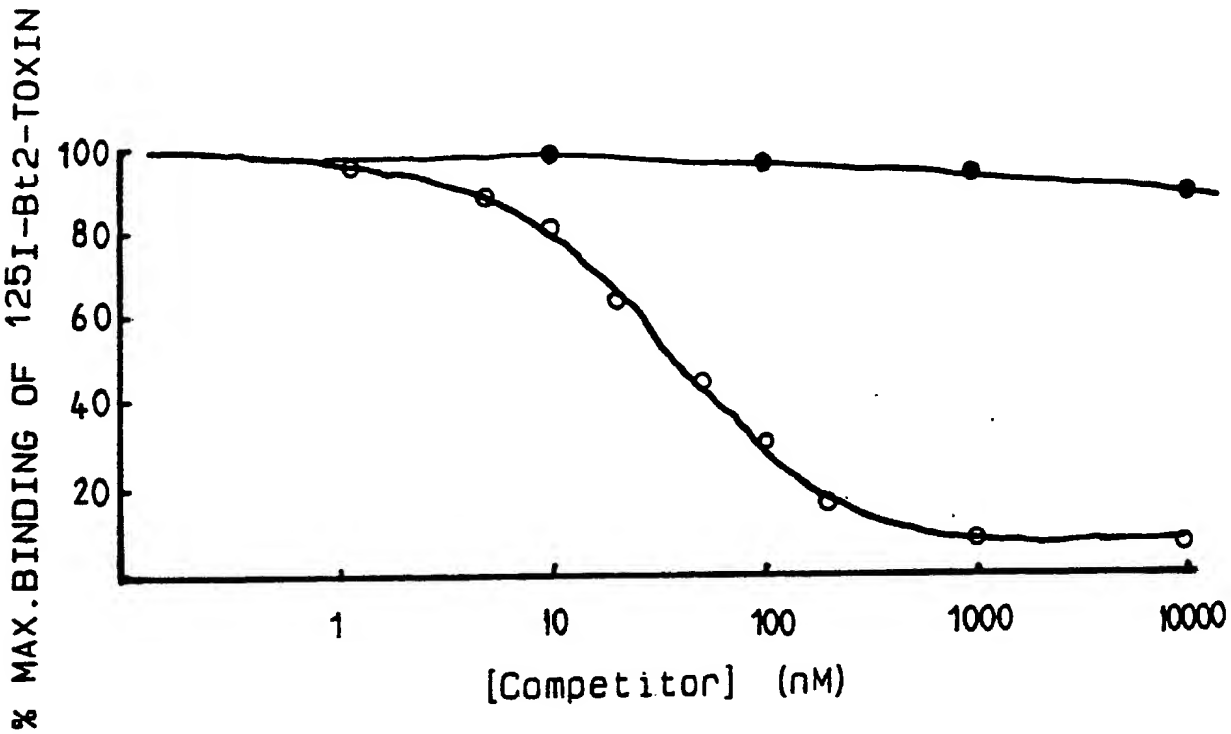

% MAX.BINDING OF 125I-Bt2-TOXIN
100
80
60
40
20
1
10
100
1000
10000
[Competitor] (nM)

Figure 12

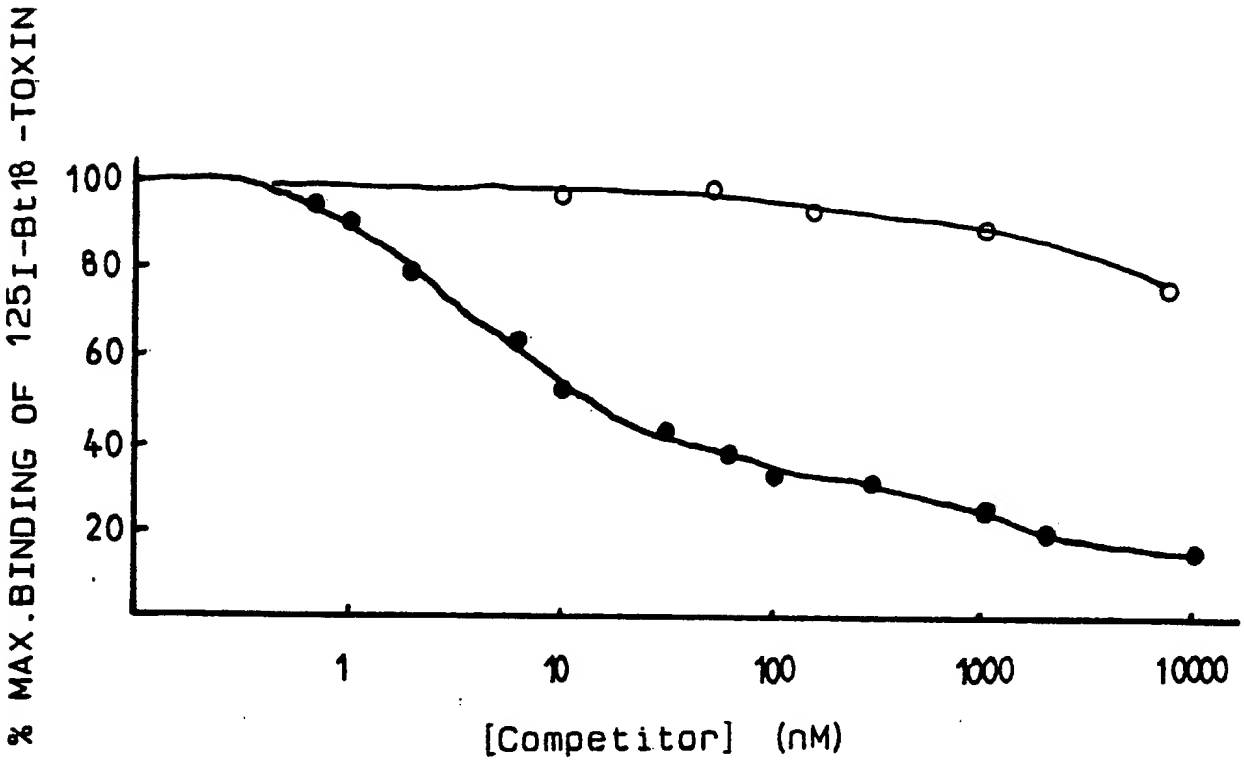

% MAX.BINDING OF 125I-Bt18 -TOXIN
100
80
60
40
20
1
10
100
1000
10000
[Competitor] (nM)

Figure 13

bt4

```
        10          20          30          40          50          60          70
GGATCTGTTT TAATATAAGG GATTTGTGCC CTTCTCGTTA TATTCTTTTA TTAGCCCCAA AAACTAGTGC

        80          90         100         110         120         130         140
AACTAAATAT TTTTATAATT ACACTGATTA AATACTTTAT TTTTGGGAGT AAGATTTATG CTGAAATGTA

       150         160         170         180         190         200         210
ATAAAATTCG TTCCATTTTC TGTATTTTCT CATAAAATGT TTCATATGCT TTAAATTGTA GTAAAGAAAA

       220         230         240         250         260         269
ACAGTACAAA CTTAAAAGGA CTTTAGTAAT TTAATAAAAA AAGGGGATAG TTT ATG GAA ATA
                                                           >
                                                           M   E   I
```

```
     278         287         296         305         314         323

AAT AAT CAA AAC CAA TGT GTG CCT TAC AAT TGT TTA AGT AAT CCT AAG GAG ATA
N   N   Q   N   Q   C   V   P   Y   N   C   L   S   N   P   K   E   I

     332         341         350         359         368         377

ATA TTA GGC GAG GAA AGG CTA GAA ACA GGG AAT ACT GTA GCA GAC ATT TCA TTA
I   L   G   E   E   R   L   E   T   G   N   T   V   A   D   I   S   L

     386         395         404         413         422         431

GGG CTT ATT AAT TTT CTA TAT TCT AAT TTT GTA CCA GGA GGA GGA TTT ATA GTA
G   L   I   N   F   L   Y   S   N   F   V   P   G   G   G   F   I   V

     440         449         458         467         476         485

GGT TTA CTA GAA TTA ATA TGG GGA TTT ATA GGG CCT TCG CAA TGG GAT ATT TTT
G   L   L   E   L   I   W   G   F   I   G   P   S   Q   W   D   I   F

     494         503         512         521         530         539

TTA GCT CAA ATT GAG CAA TTG ATT AGT CAA AGA ATA GAA GAA TTT GCT AGG AAT
L   A   Q   I   E   Q   L   I   S   Q   R   I   E   E   F   A   R   N

     548         557         566         575         584         593

CAG GCA ATT TCA AGA TTG GAG GGG CTA AGC AAT CTT TAT AAG GTC TAT GTT AGA
Q   A   I   S   R   L   E   G   L   S   N   L   Y   K   V   Y   V   R

     602         611         620         629         638         647

GCG TTT AGC GAC TGG GAG AAA GAT CCT ACT AAT CCT GCT TTA AGG GAA GAA ATG
A   F   S   D   W   E   K   D   P   T   N   P   A   L   R   E   E   M

     656         665         674         683         692         701

CGT ATA CAA TTT AAT GAC ATG AAT AGT GCT CTC ATA ACG GCT ATT CCA CTT TTT
R   I   Q   F   N   D   M   N   S   A   L   I   T   A   I   P   L   F

     710         719         728         737         746         755

AGA GTT CAA AAT TAT GAA GTT GCT CTT TTA TCT GTA TAT GTT CAA GCC GCA AAC
R   V   Q   N   Y   E   V   A   L   L   S   V   Y   V   Q   A   A   N

     764         773         782         791         800         809

TTA CAT TTA TCT ATT TTA AGG GAT GTT TCA GTT TTC GGA GAA AGA TGG GGA TAT
L   H   L   S   I   L   R   D   V   S   V   F   G   E   R   W   G   Y

     818         827         836         845         854         863

GAT ACA GCG ACT ATC AAT AAT CGC TAT AGT GAT CTG ACT AGC CTT ATT CAT GTT
D   T   A   T   I   N   N   R   Y   S   D   L   T   S   L   I   H   V

     872         881         890         899         908         917

TAT ACT AAC CAT TGT GTG GAT ACG TAT AAT CAG GGA TTA AGG CGT TTG GAA GGT
Y   T   N   H   C   V   D   T   Y   N   Q   G   L   R   R   L   E   G

     926         935         944         953         962         971

CGT TTT CTT AGC GAT TGG ATT GTA TAT AAT CGT TTC CGG AGA CAA TTG ACA ATT
R   F   L   S   D   W   I   V   Y   N   R   F   R   R   Q   L   T   I

     980         989         998        1007        1016        1025
```

```
TCA GTA TTA GAT ATT GTT GCG TTT TTT CCA AAT TAT GAT ATT AGA ACA TAT CCA
S   V   L   D   I   V   A   F   F   P   N   Y   D   I   R   T   Y   P

   1034        1043        1052        1061        1070        1079

ATT CAA ACA GCT ACT CAG CTA ACG AGG GAA GTC TAT CTG GAT TTA CCT TTT ATT
I   Q   T   A   T   Q   L   T   R   E   V   Y   L   D   L   P   F   I

   1088        1097        1106        1115        1124        1133

AAT GAA AAT CTT TCT CCT GCA GCA AGC TAT CCA ACC TTT TCA GCT GCT GAA AGT
N   E   N   L   S   P   A   A   S   Y   P   T   F   S   A   A   E   S

   1142        1151        1160        1169        1178        1187

GCT ATA ATT AGA AGT CCT CAT TTA GTA GAC TTT TTA AAT AGC TTT ACC ATT TAT
A   I   I   R   S   P   H   L   V   D   F   L   N   S   F   T   I   Y

   1196        1205        1214        1223        1232        1241

ACA GAT AGT CTG GCA CGT TAT GCA TAT TGG GGA GGG CAC TTG GTA AAT TCT TTC
T   D   S   L   A   R   Y   A   Y   W   G   G   H   L   V   N   S   F

   1250        1259        1268        1277        1286        1295

CGC ACA GGA ACC ACT ACT AAT TTG ATA AGA TCC CCT TTA TAT GGA AGG GAA GGA
R   T   G   T   T   T   N   L   I   R   S   P   L   Y   G   R   E   G

   1304        1313        1322        1331        1340        1349

AAT ACA GAG CGC CCC GTA ACT ATT ACC GCA TCA CCT AGC GTA CCA ATA TTT AGA
N   T   E   R   P   V   T   I   T   A   S   P   S   V   P   I   F   R

   1358        1367        1376        1385        1394        1403

ACA CTT TCA TAT ATT ACA GGC CTT GAC AAT TCA AAT CCT GTA GCT GGA ATC GAG
T   L   S   Y   I   T   G   L   D   N   S   N   P   V   A   G   I   E

   1412        1421        1430        1439        1448        1457

GGA GTG GAA TTC CAA AAT ACT ATA AGT AGA AGT ATC TAT CGT AAA AGC GGT CCA
G   V   E   F   Q   N   T   I   S   R   S   I   Y   R   K   S   G   P

   1466        1475        1484        1493        1502        1511

ATA GAT TCT TTT AGT GAA TTA CCA CCT CAA GAT GCC AGC GTA TCT CCT GCA ATT
I   D   S   F   S   E   L   P   P   Q   D   A   S   V   S   P   A   I

   1520        1529        1538        1547        1556        1565

GGG TAT AGT CAC CGT TTA TGC CAT GCA ACA TTT TTA GAA CGG ATT AGT GGA CCA
G   Y   S   H   R   L   C   H   A   T   F   L   E   R   I   S   G   P

   1574        1583        1592        1601        1610        1619

AGA ATA GCA GGC ACC GTA TTT TCT TGG ACA CAC CGT AGT GCC AGC CCT ACT AAT
R   I   A   G   T   V   F   S   W   T   H   R   S   A   S   P   T   N

   1628        1637        1646        1655        1664        1673

GAA GTA AGT CCA TCT AGA ATT ACA CAA ATT CCA TGG GTA AAG GCG CAT ACT CTT
E   V   S   P   S   R   I   T   Q   I   P   W   V   K   A   H   T   L

   1682        1691        1700        1709        1718        1727
```

```
GCA TCT GGT GCC TCC GTC ATT AAA GGT CCT GGA TTT ACA GGT GGA GAT ATT CTG
A   S   G   A   S   V   I   K   G   P   G   F   T   G   G   D   I   L

   1736        1745        1754        1763        1772        1781

ACT AGG AAT AGT ATG GGC GAG CTG GGG ACC TTA CGA GTA ACC TTC ACA GGA AGA
T   R   N   S   M   G   E   L   G   T   L   R   V   T   F   T   G   R

   1790        1799        1808        1817        1826        1835

TTA CCA CAA AGT TAT TAT ATA CGT TTC CGT TAT GCT TCG GTA GCA AAT AGG AGT
L   P   Q   S   Y   Y   I   R   F   R   Y   A   S   V   A   N   R   S

   1844        1853        1862        1871        1880        1889

GGT ACA TTT AGA TAT TCA CAG CCA CCT TCG TAT GGA ATT TCA TTT CCA AAA ACT
G   T   F   R   Y   S   Q   P   P   S   Y   G   I   S   F   P   K   T

   1898        1907        1916        1925        1934        1943

ATG GAC GCA GGT GAA CCA CTA ACA TCT CGT TCG TTC GCT CAT ACA ACA CTC TTC
M   D   A   G   E   P   L   T   S   R   S   F   A   H   T   T   L   F

   1952        1961        1970        1979        1988        1997

ACT CCA ATA ACC TTT TCA CGA GCT CAA GAA GAA TTT GAT CTA TAC ATC CAA TCG
T   P   I   T   F   S   R   A   Q   E   E   F   D   L   Y   I   Q   S

   2006        2015        2024        2033        2042        2051

GGT GTT TAT ATA GAT CGA ATT GAA TTT ATA CCG GTT ACT GCA ACA TTT GAG GCA
G   V   Y   I   D   R   I   E   F   I   P   V   T   A   T   F   E   A

   2060        2069        2078        2087        2096        2105

GAA TAT GAT TTA GAA AGA GCG CAA AAG GTG GTG AAT GCC CTG TTT ACG TCT ACA
E   Y   D   L   E   R   A   Q   K   V   V   N   A   L   F   T   S   T

   2114        2123        2132        2141        2150        2159

AAC CAA CTA GGG CTA AAA ACA GAT GTG ACG GAT TAT CAT ATT GAT CAG GTA TCC
N   Q   L   G   L   K   T   D   V   T   D   Y   H   I   D   Q   V   S

   2168        2177        2186        2195        2204        2213

AAT CTA GTT GCG TGT TTA TCG GAT GAA TTT TGT CTG GAT GAA AAG AGA GAA TTG
N   L   V   A   C   L   S   D   E   F   C   L   D   E   K   R   E   L

   2222        2231        2240        2249        2258        2267

TCC GAG AAA GTT AAA CAT GCA AAG CGA CTC AGT GAT GAG CGG AAT TTA CTT CAA
S   E   K   V   K   H   A   K   R   L   S   D   E   R   N   L   L   Q

   2276        2285        2294        2303        2312        2321

GAT CCA AAC TTC AGA GGG ATC AAT AGG CAA CCA GAC CGT GGC TGG AGA GGA AGT
D   P   N   F   R   G   I   N   R   Q   P   D   R   G   W   R   G   S

   2330        2339        2348        2357        2366        2375

ACG GAT ATT ACT ATC CAA GGA GGA GAT GAC GTA TTC AAA GAG AAT TAC GTT ACG
T   D   I   T   I   Q   G   G   D   D   V   F   K   E   N   Y   V   T

   2384        2393        2402        2411        2420        2429
```

Fig. 2 / Suite

```
CTA CCG GGT ACC TTT GAT GAG TGC TAT CCA ACG TAT TTA TAT CAA AAA ATA GAT
L   P   G   T   F   D   E   C   Y   P   T   Y   L   Y   Q   K   I   D

   2438        2447        2456        2465        2474        2483

GAG TCG AAA TTA AAA GCC TAT ACC CGT TAT CAA TTA AGA GGG TAT ATC GAA GAT
E   S   K   L   K   A   Y   T   R   Y   Q   L   R   G   Y   I   E   D

   2492        2501        2510        2519        2528        2537

AGT CAA GAC TTA GAA ATC TAT TTA ATT CGT TAC AAT GCA AAA CAC GAA ATA GTA
S   Q   D   L   E   I   Y   L   I   R   Y   N   A   K   H   E   I   V

   2546        2555        2564        2573        2582        2591

AAT GTA CCA GGT ACA GGA AGT TTA TGG CCT CTT TCT GTA GAA AAT CAA ATT GGA
N   V   P   G   T   G   S   L   W   P   L   S   V   E   N   Q   I   G

   2600        2609        2618        2627        2636        2645

CCT TGT GGA GAA CCG AAT CGA TGC GCG CCA CAC CTT GAA TGG AAT CCT GAT TTA
P   C   G   E   P   N   R   C   A   P   H   L   E   W   N   P   D   L

   2654        2663        2672        2681        2690        2699

CAC TGT TCC TGC AGA GAC GGG GAA AAA TGT GCA CAT CAT TCT CAT CAT TTC TCT
H   C   S   C   R   D   G   E   K   C   A   H   H   S   H   H   F   S

   2708        2717        2726        2735        2744        2753

TTG GAC ATT GAT GTT GGA TGT ACA GAC TTA AAT GAG GAC TTA GGT GTA TGG GTG
L   D   I   D   V   G   C   T   D   L   N   E   D   L   G   V   W   V

   2762        2771        2780        2789        2798        2807

ATA TTC AAG ATT AAG ACG CAA GAT GGC CAC GCA CGA CTA GGG AAT CTA GAG TTT
I   F   K   I   K   T   Q   D   G   H   A   R   L   G   N   L   E   F

   2816        2825        2834        2843        2852        2861

CTC GAA GAG AAA CCA TTA TTA GGA GAA GCA CTA GCT CGT GTG AAA AGA GCG GAG
L   E   E   K   P   L   L   G   E   A   L   A   R   V   K   R   A   E

   2870        2879        2888        2897        2906        2915

AAA AAA TGG AGA GAC AAA CGC GAA ACA TTA CAA TTG GAA ACA ACT ATC GTT TAT
K   K   W   R   D   K   R   E   T   L   Q   L   E   T   T   I   V   Y

   2924        2933        2942        2951        2960        2969

AAA GAG GCA AAA GAA TCT GTA GAT GCT TTA TTT GTA AAC TCT CAA TAT GAT AGA
K   E   A   K   E   S   V   D   A   L   F   V   N   S   Q   Y   D   R

   2978        2987        2996        3005        3014        3023

TTA CAA GCG GAT ACG AAC ATC GCG ATG ATT CAT GCG GCA GAT AAA CGC GTT CAT
L   Q   A   D   T   N   I   A   M   I   H   A   A   D   K   R   V   H

   3032        3041        3050        3059        3068        3077

AGA ATT CGA GAA GCG TAT CTG CCG GAG CTG TCT GTG ATT CCG GGT GTC AAT GCG
R   I   R   E   A   Y   L   P   E   L   S   V   I   P   G   V   N   A

   3086        3095        3104        3113        3122        3131
```

Fig 12/SUITE

```
GCT ATT TTT GAA GAA TTA GAA GAG CGT ATT TTC ACT GCA TTT TCC CTA TAT GAT
A   I   F   E   E   L   E   E   R   I   F   T   A   F   S   L   Y   D

   3140        3149        3158        3167        3176        3185

GCG AGA AAT ATT ATT AAA AAT GGC GAT TTC AAT AAT GGC TTA TTA TGC TGG AAC
A   R   N   I   I   K   N   G   D   F   N   N   G   L   L   C   W   N

   3194        3203        3212        3221        3230        3239

GTG AAA GGG CAT GTA GAG GTA GAA GAA CAA AAC AAT CAC CGT TCA GTC CTG GTT
V   K   G   H   V   E   V   E   E   Q   N   N   H   R   S   V   L   V

   3248        3257        3266        3275        3284        3293

ATC CCA GAA TGG GAG GCA GAA GTG TCA CAA GAG GTT CGT GTC TGT CCA GGT CGT
I   P   E   W   E   A   E   V   S   Q   E   V   R   V   C   P   G   R

   3302        3311        3320        3329        3338        3347

GGC TAT ATC CTT CGT GTT ACA GCG TAC AAA GAG GGA TAT GGA GAA GGT TGC GTA
G   Y   I   L   R   V   T   A   Y   K   E   G   Y   G   E   G   C   V

   3356        3365        3374        3383        3392        3401

ACG ATC CAT GAG ATC GAG AAC AAT ACA GAC GAA CTG AAA TTC AAC AAC TGT GTA
T   I   H   E   I   E   N   N   T   D   E   L   K   F   N   N   C   V

   3410        3419        3428        3437        3446        3455

GAA GAG GAA GTA TAT CCA AAC AAC ACG GTA ACG TGT ATT AAT TAT ACT GCG ACT
E   E   E   V   Y   P   N   N   T   V   T   C   I   N   Y   T   A   T

   3464        3473        3482        3491        3500        3509

CAA GAA GAA TAT GAG GGT ACG TAC ACT TCT CGT AAT CGA GGA TAT GAC GAA GCC
Q   E   E   Y   E   G   T   Y   T   S   R   N   R   G   Y   D   E   A

   3518        3527        3536        3545        3554        3563

TAT GGT AAT AAC CCT TCC GTA CCA GCT GAT TAT GCG TCA GTC TAT GAA GAA AAA
Y   G   N   N   P   S   V   P   A   D   Y   A   S   V   Y   E   E   K

   3572        3581        3590        3599        3608        3617

TCG TAT ACA GAT AGA CGA AGA GAG AAT CCT TGT GAA TCT AAC AGA GGA TAT GGA
S   Y   T   D   R   R   R   E   N   P   C   E   S   N   R   G   Y   G

   3626        3635        3644        3653        3662        3671

GAT TAC ACA CCA CTA CCA GCT GGT TAT GTA ACA AAG GAA TTA GAG TAC TTC CCA
D   Y   T   P   L   P   A   G   Y   V   T   K   E   L   E   Y   F   P

   3680        3689        3698        3707        3716        3725

GAG ACC GAT AAG GTA TGG ATT GAG ATT GGA GAA ACA GAA GGA ACA TTC ATC GTG
E   T   D   K   V   W   I   E   I   G   E   T   E   G   T   F   I   V

   3734        3743        3752        3761        3771        3781        3791
                                       >
GAC AGC GTG GAA TTA CTC CTT ATG GAG GAA TAG GACCATCCGA GTATAGCAGT TTAATAAATA
D   S   V   E   L   L   L   M   E   E   .

      3801        3811        3821        3831        3841        3851        3861
```

TTAATTAAAA TAGTAGTCTA ACTTCCGTTC CAATTAAATA AGTAAATTAC AGTTGTAAAA AAAAACGAAC

3871 3881 3891 3901

ATTACTCTTC AAAGAGCGAT GTCCGTTTTT TATATGGTGT GT

SUITE

Figure 14

bt15

```
        10         20         30         40         50         60         70
AATAGAATCT CAAATCTCGA TGACTGCTTA GTCTTTTTAA TACTGTCTAC TTGACAGGGG TAGGAACATA

        80         90        100        110        120        130        140
ATCGGTCAAT TTTAAATATG GGGCATATAT TGATATTTTA TAAAATTTGT TACGTTTTTT GTATTTTTTC

       150        160        170        180        190        200        210
ATAAGATGTG TCATATGTAT TAAATCGTGG TAATGAAAAA CAGTATCAAA CTATCAGAAC TTTGGTAGTT

       220        230        239        248        257        266
                             >
TAATAAAAAA ACGGAGGTAT TTT ATG GAG GAA AAT AAT CAA AAT CAA TGC ATA CCT
                          MET Glu Glu Asn Asn Gln Asn Gln Cys Ile Pro

       275        284        293        302        311        320
TAC AAT TGT TTA AGT AAT CCT GAA GAA GTA CTT TTG GAT GGA GAA CGG ATA TCA
Tyr Asn Cys Leu Ser Asn Pro Glu Glu Val Leu Leu Asp Gly Glu Arg Ile Ser

       329        338        347        356        365        374
ACT GGT AAT TCA TCA ATT GAT ATT TCT CTG TCA CTT GTT CAG TTT CTG GTA TCT
Thr Gly Asn Ser Ser Ile Asp Ile Ser Leu Ser Leu Val Gln Phe Leu Val Ser
```

```
        383         392         401         410         419         428

AAC TTT GTA CCA GGG GGA GGA TTT TTA GTT GGA TTA ATA GAT TTT GTA TGG GGA
Asn Phe Val Pro Gly Gly Gly Phe Leu Val Gly Leu Ile Asp Phe Val Trp Gly

        437         446         455         464         473         482

ATA GTT GGC CCT TCT CAA TGG GAT GCA TTT CTA GTA CAA ATT GAA CAA TTA ATT
Ile Val Gly Pro Ser Gln Trp Asp Ala Phe Leu Val Gln Ile Glu Gln Leu Ile

        491         500         509         518         527         536

AAT GAA AGA ATA GCT GAA TTT GCT AGG AAT GCT GCT ATT GCT AAT TTA GAA GGA
Asn Glu Arg Ile Ala Glu Phe Ala Arg Asn Ala Ala Ile Ala Asn Leu Glu Gly

        545         554         563         572         581         590

TTA GGA AAC AAT TTC AAT ATA TAT GTG GAA GCA TTT AAA GAA TGG GAA GAA GAT
Leu Gly Asn Asn Phe Asn Ile Tyr Val Glu Ala Phe Lys Glu Trp Glu Glu Asp

        599         608         617         626         635         644

CCT AAT AAT CCA GAA ACC AGG ACC AGA GTA ATT GAT CGC TTT CGT ATA CTT GAT
Pro Asn Asn Pro Glu Thr Arg Thr Arg Val Ile Asp Arg Phe Arg Ile Leu Asp

        653         662         671         680         689         698

GGG CTA CTT GAA AGG GAC ATT CCT TCG TTT CGA ATT TCT GGA TTT GAA GTA CCC
Gly Leu Leu Glu Arg Asp Ile Pro Ser Phe Arg Ile Ser Gly Phe Glu Val Pro

        707         716         725         734         743         752

CTT TTA TCC GTT TAT GCT CAA GCG GCC AAT CTG CAT CTA GCT ATA TTA AGA GAT
Leu Leu Ser Val Tyr Ala Gln Ala Ala Asn Leu His Leu Ala Ile Leu Arg Asp

        761         770         779         788         797         806

TCT GTA ATT TTT GGA GAA AGA TGG GGA TTG ACA ACG ATA AAT GTC AAT GAA AAC
Ser Val Ile Phe Gly Glu Arg Trp Gly Leu Thr Thr Ile Asn Val Asn Glu Asn

        815         824         833         842         851         860

TAT AAT AGA CTA ATT AGG CAT ATT GAT GAA TAT GCT GAT CAC TGT GCA AAT ACG
Tyr Asn Arg Leu Ile Arg His Ile Asp Glu Tyr Ala Asp His Cys Ala Asn Thr

        869         878         887         896         905         914

TAT AAT CGG GGA TTA AAT AAT TTA CCG AAA TCT ACG TAT CAA GAT TGG ATA ACA
Tyr Asn Arg Gly Leu Asn Asn Leu Pro Lys Ser Thr Tyr Gln Asp Trp Ile Thr

        923         932         941         950         959         968

TAT AAT CGA TTA CGG AGA GAC TTA ACA TTG ACT GTA TTA GAT ATC GCC GCT TTC
Tyr Asn Arg Leu Arg Arg Asp Leu Thr Leu Thr Val Leu Asp Ile Ala Ala Phe

        977         986         995        1004        1013        1022

TTT CCA AAC TAT GAC AAT AGG AGA TAT CCA ATT CAG CCA GTT GGT CAA CTA ACA
Phe Pro Asn Tyr Asp Asn Arg Arg Tyr Pro Ile Gln Pro Val Gly Gln Leu Thr

       1031        1040        1049        1058        1067        1076

AGG GAA GTT TAT ACG GAC CCA TTA ATT AAT TTT AAT CCA CAG TTA CAG TCT GTA
Arg Glu Val Tyr Thr Asp Pro Leu Ile Asn Phe Asn Pro Gln Leu Gln Ser Val

       1085        1094        1103        1112        1121        1130
```

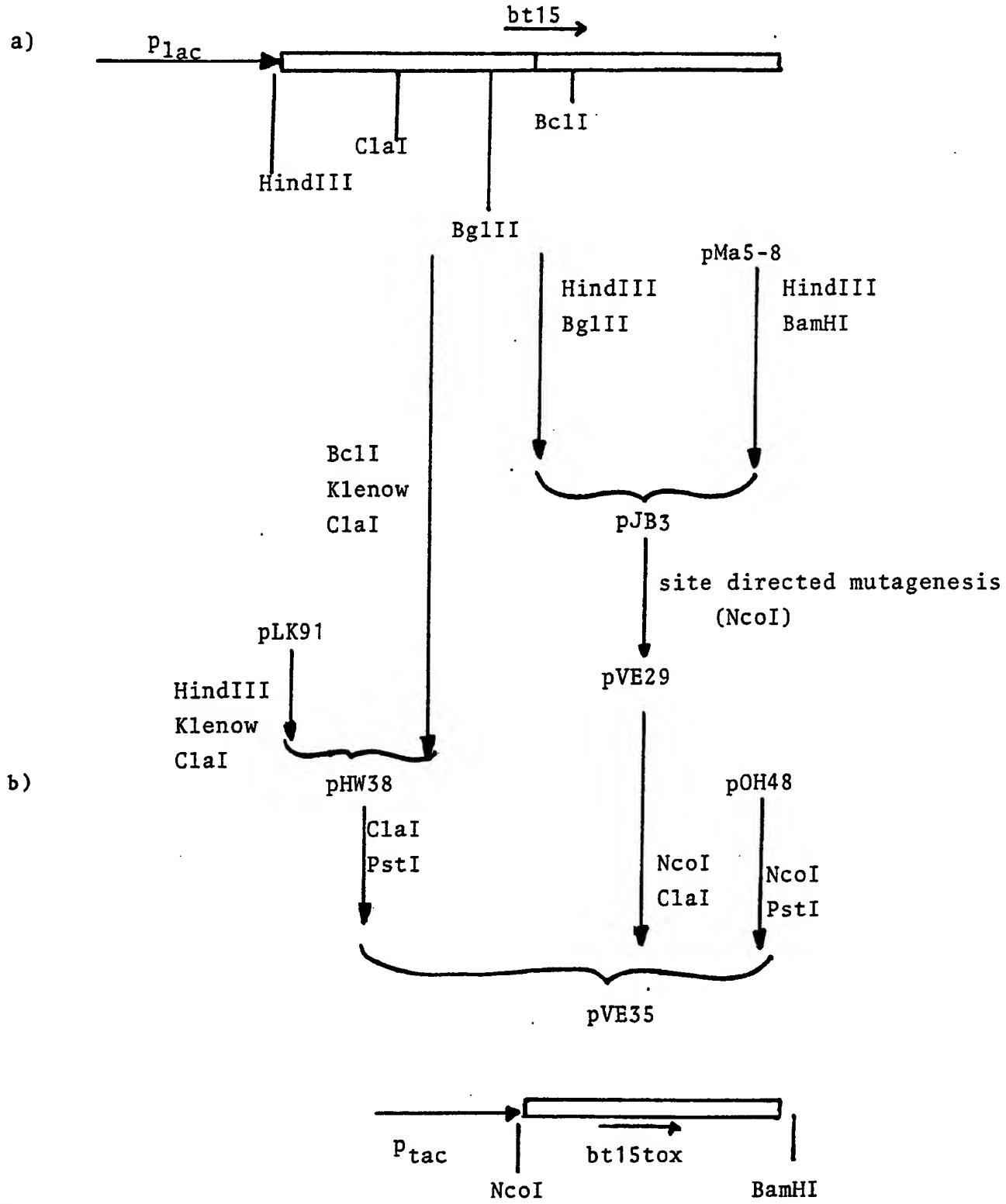
```
GCT CAA TTA CCT ACT TTT AAC GTT ATG GAG AGC AGC GCA ATT AGA AAT CCT CAT
Ala Gln Leu Pro Thr Phe Asn Val MET Glu Ser Ser Ala Ile Arg Asn Pro His

      1139        1148        1157        1166        1175        1184

TTA TTT GAT ATA TTG AAT AAT CTT ACA ATC TTT ACG GAT TGG TTT AGT GTT GGA
Leu Phe Asp Ile Leu Asn Asn Leu Thr Ile Phe Thr Asp Trp Phe Ser Val Gly

      1193        1202        1211        1220        1229        1238

CGC AAT TTT TAT TGG GGA GGA CAT CGA GTA ATA TCT AGC CTT ATA GGA GGT GGT
Arg Asn Phe Tyr Trp Gly Gly His Arg Val Ile Ser Ser Leu Ile Gly Gly Gly

      1247        1256        1265        1274        1283        1292

AAC ATA ACA TCT CCT ATA TAT GGA AGA GAG GCG AAC CAG GAG CCT CCA AGA TCC
Asn Ile Thr Ser Pro Ile Tyr Gly Arg Glu Ala Asn Gln Glu Pro Pro Arg Ser

      1301        1310        1319        1328        1337        1346

TTT ACT TTT AAT GGA CCG GTA TTT AGG ACT TTA TCA AAT CCT ACT TTA CGA TTA
Phe Thr Phe Asn Gly Pro Val Phe Arg Thr Leu Ser Asn Pro Thr Leu Arg Leu

      1355        1364        1373        1382        1391        1400

TTA CAG CAA CCT TGG CCA GCG CCA CCA TTT AAT TTA CGT GGT GTT GAA GGA GTA
Leu Gln Gln Pro Trp Pro Ala Pro Pro Phe Asn Leu Arg Gly Val Glu Gly Val

      1409        1418        1427        1436        1445        1454

GAA TTT TCT ACA CCT ACA AAT AGC TTT ACG TAT CGA GGA AGA GGT ACG GTT GAT
Glu Phe Ser Thr Pro Thr Asn Ser Phe Thr Tyr Arg Gly Arg Gly Thr Val Asp

      1463        1472        1481        1490        1499        1508

TCT TTA ACT GAA TTA CCG CCT GAG GAT AAT AGT GTG CCA CCT CGC GAA GGA TAT
Ser Leu Thr Glu Leu Pro Pro Glu Asp Asn Ser Val Pro Pro Arg Glu Gly Tyr

      1517        1526        1535        1544        1553        1562

AGT CAT CGT TTA TGT CAT GCA ACT TTT GTT CAA AGA TCT GGA ACA CCT TTT TTA
Ser His Arg Leu Cys His Ala Thr Phe Val Gln Arg Ser Gly Thr Pro Phe Leu

      1571        1580        1589        1598        1607        1616

ACA ACT GGT GTA GTA TTT TCT TGG ACG CAT CGT AGT GCA ACT CTT ACA AAT ACA
Thr Thr Gly Val Val Phe Ser Trp Thr His Arg Ser Ala Thr Leu Thr Asn Thr

      1625        1634        1643        1652        1661        1670

ATT GAT CCA GAG AGA ATT AAT CAA ATA CCT TTA GTG AAA GGA TTT AGA GTT TGG
Ile Asp Pro Glu Arg Ile Asn Gln Ile Pro Leu Val Lys Gly Phe Arg Val Trp

      1679        1688        1697        1706        1715        1724

GGG GGC ACC TCT GTC ATT ACA GGA CCA GGA TTT ACA GGA GGG GAT ATC CTT CGA
Gly Gly Thr Ser Val Ile Thr Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu Arg

      1733        1742        1751        1760        1769        1778

AGA AAT ACC TTT GGT GAT TTT GTA TCT CTA CAA GTC AAT ATT AAT TCA CCA ATT
Arg Asn Thr Phe Gly Asp Phe Val Ser Leu Gln Val Asn Ile Asn Ser Pro Ile

      1787        1796        1805        1814        1823        1832
```

```
ACC CAA AGA TAC CGT TTA AGA TTT CGT TAC GCT TCC AGT AGG GAT GCA CGA GTT
Thr Gln Arg Tyr Arg Leu Arg Phe Arg Tyr Ala Ser Ser Arg Asp Ala Arg Val

      1841        1850        1859        1868        1877        1886

ATA GTA TTA ACA GGA GCG GCA TCC ACA GGA GTG GGA GGC CAA GTT AGT GTA AAT
Ile Val Leu Thr Gly Ala Ala Ser Thr Gly Val Gly Gly Gln Val Ser Val Asn

      1895        1904        1913        1922        1931        1940

ATG CCT CTT CAG AAA ACT ATG GAA ATA GGG GAG AAC TTA ACA TCT AGA ACA TTT
MET Pro Leu Gln Lys Thr MET Glu Ile Gly Glu Asn Leu Thr Ser Arg Thr Phe

      1949        1958        1967        1976        1985        1994

AGA TAT ACC GAT TTT AGT AAT CCT TTT TCA TTT AGA GCT AAT CCA GAT ATA ATT
Arg Tyr Thr Asp Phe Ser Asn Pro Phe Ser Phe Arg Ala Asn Pro Asp Ile Ile

      2003        2012        2021        2030        2039        2048

GGG ATA AGT GAA CAA CCT CTA TTT GGT GCA GGT TCT ATT AGT AGC GGT GAA CTT
Gly Ile Ser Glu Gln Pro Leu Phe Gly Ala Gly Ser Ile Ser Ser Gly Glu Leu

      2057        2066        2075        2084        2093        2102

TAT ATA GAT AAA ATT GAA ATT ATT CTA GCA GAT GCA ACA TTT GAA GCA GAA TCT
Tyr Ile Asp Lys Ile Glu Ile Ile Leu Ala Asp Ala Thr Phe Glu Ala Glu Ser

      2111        2120        2129        2138        2147        2156

GAT TTA GAA AGA GCA CAA AAG GCG GTG AAT GCC CTG TTT ACT TCT TCC AAT CAA
Asp Leu Glu Arg Ala Gln Lys Ala Val Asn Ala Leu Phe Thr Ser Ser Asn Gln

      2165        2174        2183        2192        2201        2210

ATC GGG TTA AAA ACC GAT GTG ACG GAT TAT CAT ATT GAT CAA GTA TCC AAT TTA
Ile Gly Leu Lys Thr Asp Val Thr Asp Tyr His Ile Asp Gln Val Ser Asn Leu

      2219        2228        2237        2246        2255        2264

GTG GAT TGT TTA TCA GAT GAA TTT TGT CTG GAT GAA AAG CGA GAA TTG TCC GAG
Val Asp Cys Leu Ser Asp Glu Phe Cys Leu Asp Glu Lys Arg Glu Leu Ser Glu

      2273        2282        2291        2300        2309        2318

AAA GTC AAA CAT GCG AAG CGA CTC AGT GAT GAG CGG AAT TTA CTT CAA GAT CCA
Lys Val Lys His Ala Lys Arg Leu Ser Asp Glu Arg Asn Leu Leu Gln Asp Pro

      2327        2336        2345        2354        2363        2372

AAC TTC AGA GGG ATC AAT AGA CAA CCA GAC CGT GGC TGG AGA GGA AGT ACA GAT
Asn Phe Arg Gly Ile Asn Arg Gln Pro Asp Arg Gly Trp Arg Gly Ser Thr Asp

      2381        2390        2399        2408        2417        2426

ATT ACC ATC CAA GGA GGA GAT GAC GTA TTC AAA GAG AAT TAC GTC ACA CTA CCG
Ile Thr Ile Gln Gly Gly Asp Asp Val Phe Lys Glu Asn Tyr Val Thr Leu Pro

      2435        2444        2453        2462        2471        2480

GGT ACC GTT GAT GAG TGC TAT CCA ACG TAT TTA TAT CAG AAA ATA GAT GAG TCG
Gly Thr Val Asp Glu Cys Tyr Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu Ser

      2489        2498        2507        2516        2525        2534
```

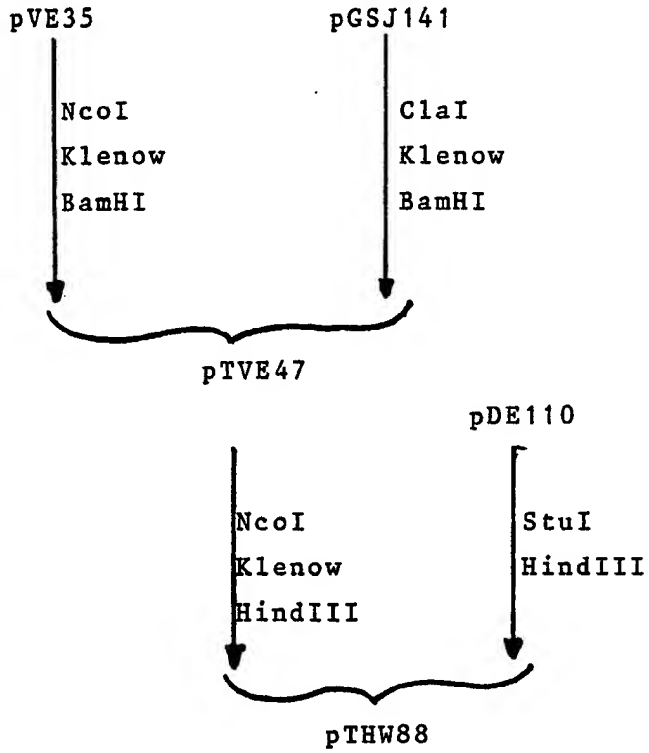
```
AAA TTA AAA GCT TAT ACC CGT TAT GAA TTA AGA GGG TAT ATC GAA GAT AGT CAA
Lys Leu Lys Ala Tyr Thr Arg Tyr Glu Leu Arg Gly Tyr Ile Glu Asp Ser Gln

      2543        2552        2561        2570        2579        2588

GAC TTA GAA ATC TAT TTG ATC CGT TAC AAT GCA AAA CAC GAA ATA GTA AAT GTG
Asp Leu Glu Ile Tyr Leu Ile Arg Tyr Asn Ala Lys His Glu Ile Val Asn Val

      2597        2606        2615        2624        2633        2642

CCA GGC ACG GGT TCC TTA TGG CCG CTT TCA GCC CAA AGT CCA ATC GGA AAG TGT
Pro Gly Thr Gly Ser Leu Trp Pro Leu Ser Ala Gln Ser Pro Ile Gly Lys Cys

      2651        2660        2669        2678        2687        2696

GGA GAA CCG AAT CGA TGC GCG CCA CAC CTT GAA TGG AAT CCT GAT CTA GAT TGT
Gly Glu Pro Asn Arg Cys Ala Pro His Leu Glu Trp Asn Pro Asp Leu Asp Cys

      2705        2714        2723        2732        2741        2750

TCC TGC AGA GAC GGG GAA AAA TGT GCA CAT CAT TCC CAT CAT TTC ACC TTG GAT
Ser Cys Arg Asp Gly Glu Lys Cys Ala His His Ser His His Phe Thr Leu Asp

      2759        2768        2777        2786        2795        2804

ATT GAT GTT GGA TGT ACA GAC TTA AAT GAG GAC TTA GGT GTA TGG GTG ATA TTC
Ile Asp Val Gly Cys Thr Asp Leu Asn Glu Asp Leu Gly Val Trp Val Ile Phe

      2813        2822        2831        2840        2849        2858

AAG ATT AAG ACG CAA GAT GGC CAT GCA AGA CTA GGG AAT CTA GAG TTT CTC GAA
Lys Ile Lys Thr Gln Asp Gly His Ala Arg Leu Gly Asn Leu Glu Phe Leu Glu

      2867        2876        2885        2894        2903        2912

GAG AAA CCA TTA TTA GGG GAA GCA CTA GCT CGT GTG AAA AGA GCG GAG AAG AAG
Glu Lys Pro Leu Leu Gly Glu Ala Leu Ala Arg Val Lys Arg Ala Glu Lys Lys

      2921        2930        2939        2948        2957        2966

TGG AGA GAC AAA CGA GAG AAA CTG CAG TTG GAA ACA AAT ATT GTT TAT AAA GAG
Trp Arg Asp Lys Arg Glu Lys Leu Gln Leu Glu Thr Asn Ile Val Tyr Lys Glu

      2975        2984        2993        3002        3011        3020

GCA AAA GAA TCT GTA GAT GCT TTA TTT GTA AAC TCT CAA TAT GAT AGA TTA CAA
Ala Lys Glu Ser Val Asp Ala Leu Phe Val Asn Ser Gln Tyr Asp Arg Leu Gln

      3029        3038        3047        3056        3065        3074

GTG GAT ACG AAC ATC GCG ATG ATT CAT GCG GCA GAT AAA CGC GTT CAT AGA ATC
Val Asp Thr Asn Ile Ala MET Ile His Ala Ala Asp Lys Arg Val His Arg Ile

      3083        3092        3101        3110        3119        3128

CGG GAA GCG TAT CTG CCA GAG TTG TCT GTG ATT CCA GGT GTC AAT GCG GCC ATT
Arg Glu Ala Tyr Leu Pro Glu Leu Ser Val Ile Pro Gly Val Asn Ala Ala Ile

      3137        3146        3155        3164        3173        3182

TTC GAA GAA TTA GAG GGA CGT ATT TTT ACA GCG TAT TCC TTA TAT GAT GCG AGA
Phe Glu Glu Leu Glu Gly Arg Ile Phe Thr Ala Tyr Ser Leu Tyr Asp Ala Arg

      3191        3200        3209        3218        3227        3236
```

```
AAT GTC ATT AAA AAT GGC GAT TTC AAT AAT GGC TTA TTA TGC TGG AAC GTG AAA
Asn Val Ile Lys Asn Gly Asp Phe Asn Asn Gly Leu Leu Cys Trp Asn Val Lys

      3245        3254        3263        3272        3281        3290

GGT CAT GTA GAT GTA GAA GAG CAA AAC AAC CAC CGT TCG GTC CTT GTT ATC CCA
Gly His Val Asp Val Glu Glu Gln Asn Asn His Arg Ser Val Leu Val Ile Pro

      3299        3308        3317        3326        3335        3344

GAA TGG GAG GCA GAA GTG TCA CAA GAG GTT CGT GTC TGT CCA GGT CGT GGC TAT
Glu Trp Glu Ala Glu Val Ser Gln Glu Val Arg Val Cys Pro Gly Arg Gly Tyr

      3353        3362        3371        3380        3389        3398

ATC CTT CGT GTC ACA GCA TAT AAA GAG GGA TAT GGA GAG GGC TGC GTA ACG ATC
Ile Leu Arg Val Thr Ala Tyr Lys Glu Gly Tyr Gly Glu Gly Cys Val Thr Ile

      3407        3416        3425        3434        3443        3452

CAT GAG ATC GAA GAC AAT ACA GAC GAA CTG AAA TTC AGC AAC TGT GTA GAA GAG
His Glu Ile Glu Asp Asn Thr Asp Glu Leu Lys Phe Ser Asn Cys Val Glu Glu

      3461        3470        3479        3488        3497        3506

GAA GTA TAT CCA AAC AAC ACA GTA ACG TGT AAT AAT TAT ACT GGG ACT CAA GAA
Glu Val Tyr Pro Asn Asn Thr Val Thr Cys Asn Asn Tyr Thr Gly Thr Gln Glu

      3515        3524        3533        3542        3551        3560

GAA TAT GAG GGT ACG TAC ACT TCT CGT AAT CAA GGA TAT GAC GAA GCC TAT GGT
Glu Tyr Glu Gly Thr Tyr Thr Ser Arg Asn Gln Gly Tyr Asp Glu Ala Tyr Gly

      3569        3578        3587        3596        3605        3614

AAT AAC CCT TCC GTA CCA GCT GAT TAC GCT TCA GTC TAT GAA GAA AAA TCG TAT
Asn Asn Pro Ser Val Pro Ala Asp Tyr Ala Ser Val Tyr Glu Glu Lys Ser Tyr

      3623        3632        3641        3650        3659        3668

ACA GAT GGA CGA AGA GAG AAT CCT TGT GAA TCT AAC AGA GGC TAT GGG GAT TAC
Thr Asp Gly Arg Arg Glu Asn Pro Cys Glu Ser Asn Arg Gly Tyr Gly Asp Tyr

      3677        3686        3695        3704        3713        3722

ACA CCA CTA CCG GCT GGT TAT GTA ACA AAG GAT TTA GAG TAC TTC CCA GAG ACC
Thr Pro Leu Pro Ala Gly Tyr Val Thr Lys Asp Leu Glu Tyr Phe Pro Glu Thr

      3731        3740        3749        3758        3767        3776

GAT AAG GTA TGG ATT GAG ATC GGA GAA ACA GAA GGA ACA TTC ATC GTG GAT AGC
Asp Lys Val Trp Ile Glu Ile Gly Glu Thr Glu Gly Thr Phe Ile Val Asp Ser

      3785        3794        3803        3813        3823        3833
                              >
GTG GAA TTA CTC CTT ATG GAG GAA TAA GATACGTTAT AAAATGTAAC GTATGCAAAT
Val Glu Leu Leu Leu MET Glu Glu  .

      3843        3853        3863        3873        3883        3893        3903

AAAGAATGAT TACTGACCTA TATTAACAGA TAAATAAGAA AATTTTTATA CGAATAAAAA ACGGACATCA

      3913        3923
```

Figure 15


a)
bt15
P_{lac}
HindIII
ClaI
BclI
BglII
pMa5-8
HindIII
BglII
HindIII
BamHI
BclI
Klenow
ClaI
pJB3
site directed mutagenesis
(NcoI)
pLK91
pVE29
HindIII
Klenow
ClaI
b)
pHW38
pOH48
ClaI
PstI
NcoI
ClaI
NcoI
PstI
pVE35
P_{tac}
bt15tox
NcoI
BamHI

c)


pVE35
pGSJ141
NcoI
Klenow
BamHI
ClaI
Klenow
BamHI
pTVE47
pDE110
NcoI
Klenow
HindIII
StuI
HindIII
pTHW88

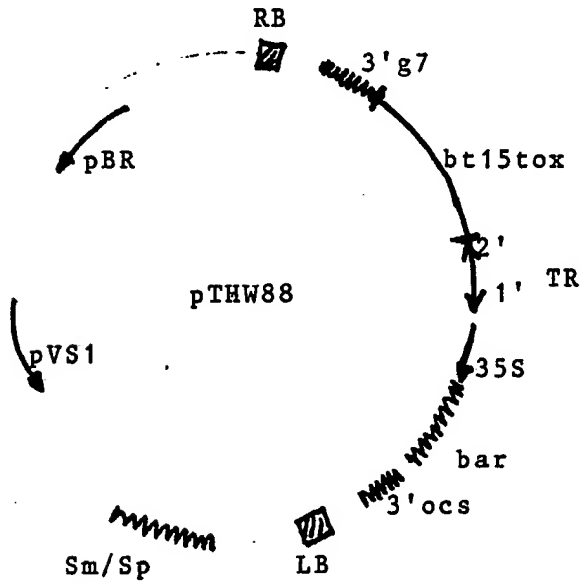
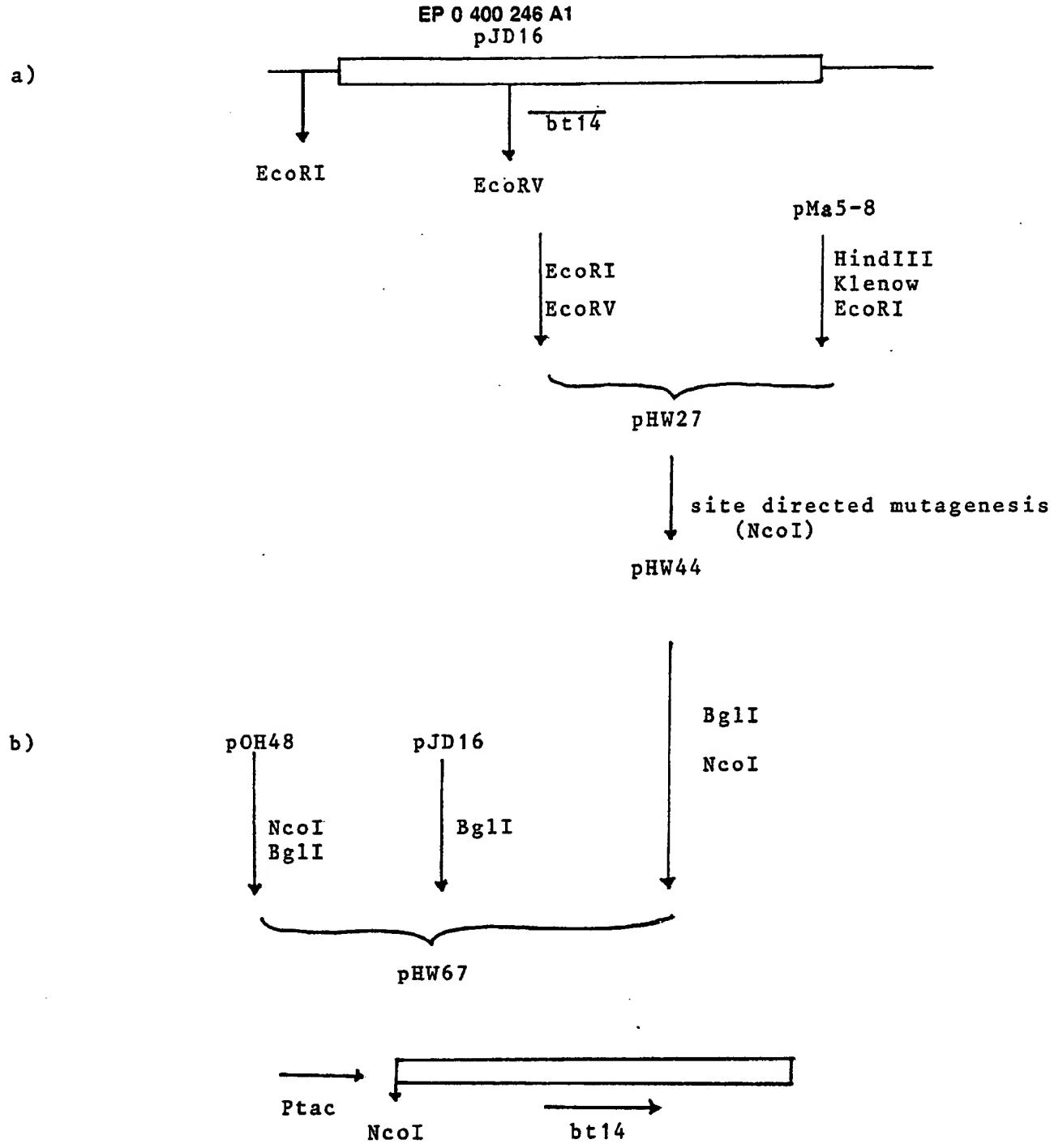
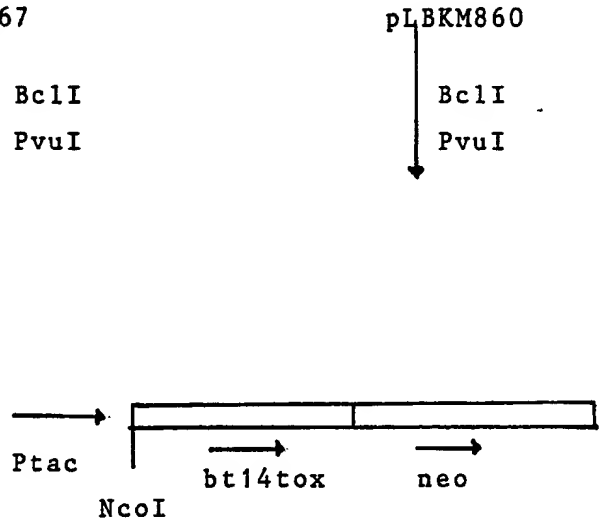

RB
3'g7
pBR
bt15tox
2'
1'
TR
pTHW88
pVS1
35S
bar
3'ocs
Sm/Sp
LB

FIG 15 -END-

Figure 16


pJD16
a)
bt14
EcoRI
EcoRV
pMa5-8
EcoRI
EcoRV
HindIII
Klenow
EcoRI
pHW27
site directed mutagenesis
(NcoI)
pHW44
b)
pOH48
pJD16
BglI
NcoI
NcoI
BglI
BglI
pHW67
Ptac
NcoI
bt14

c)



d)

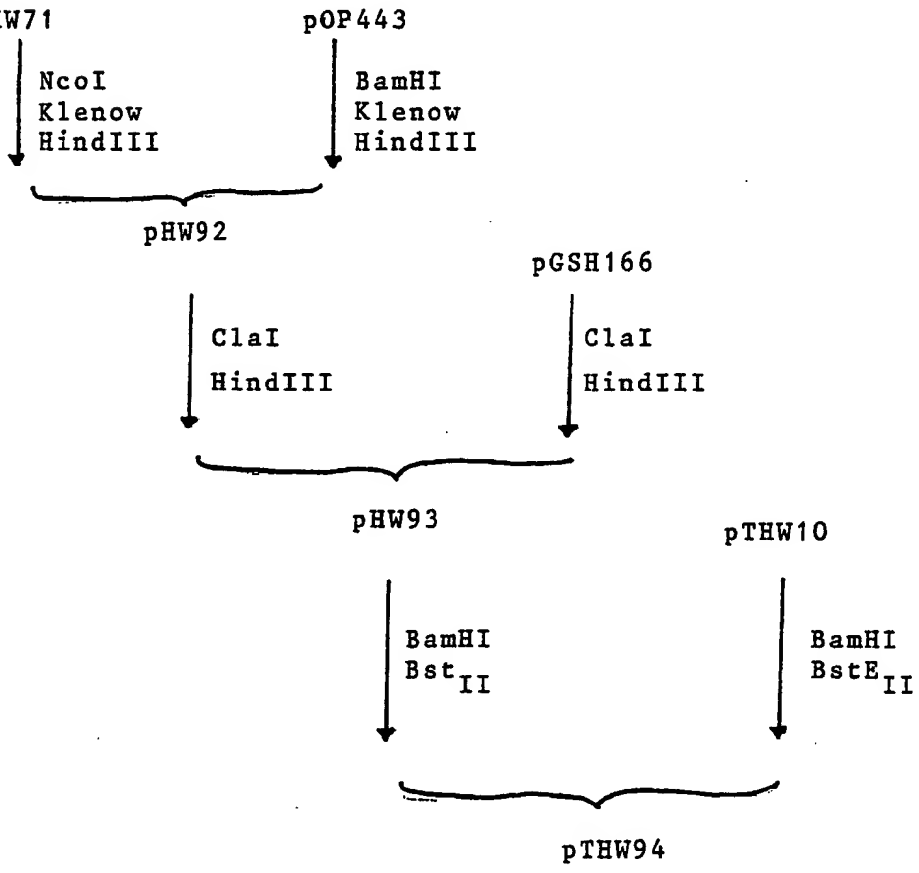


FIG 1 / sheet

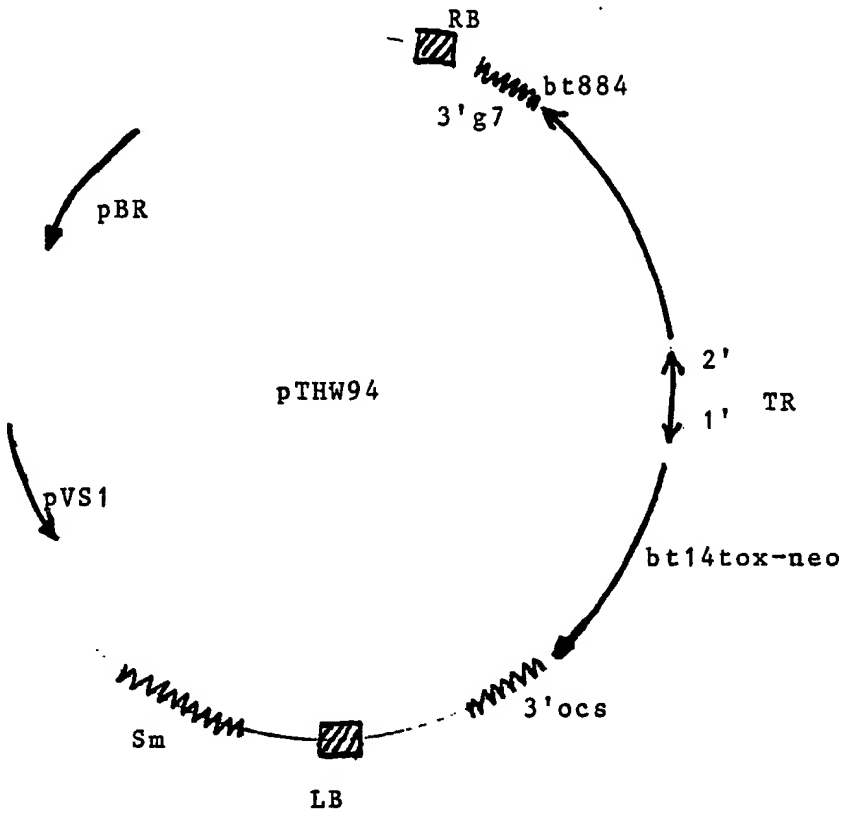
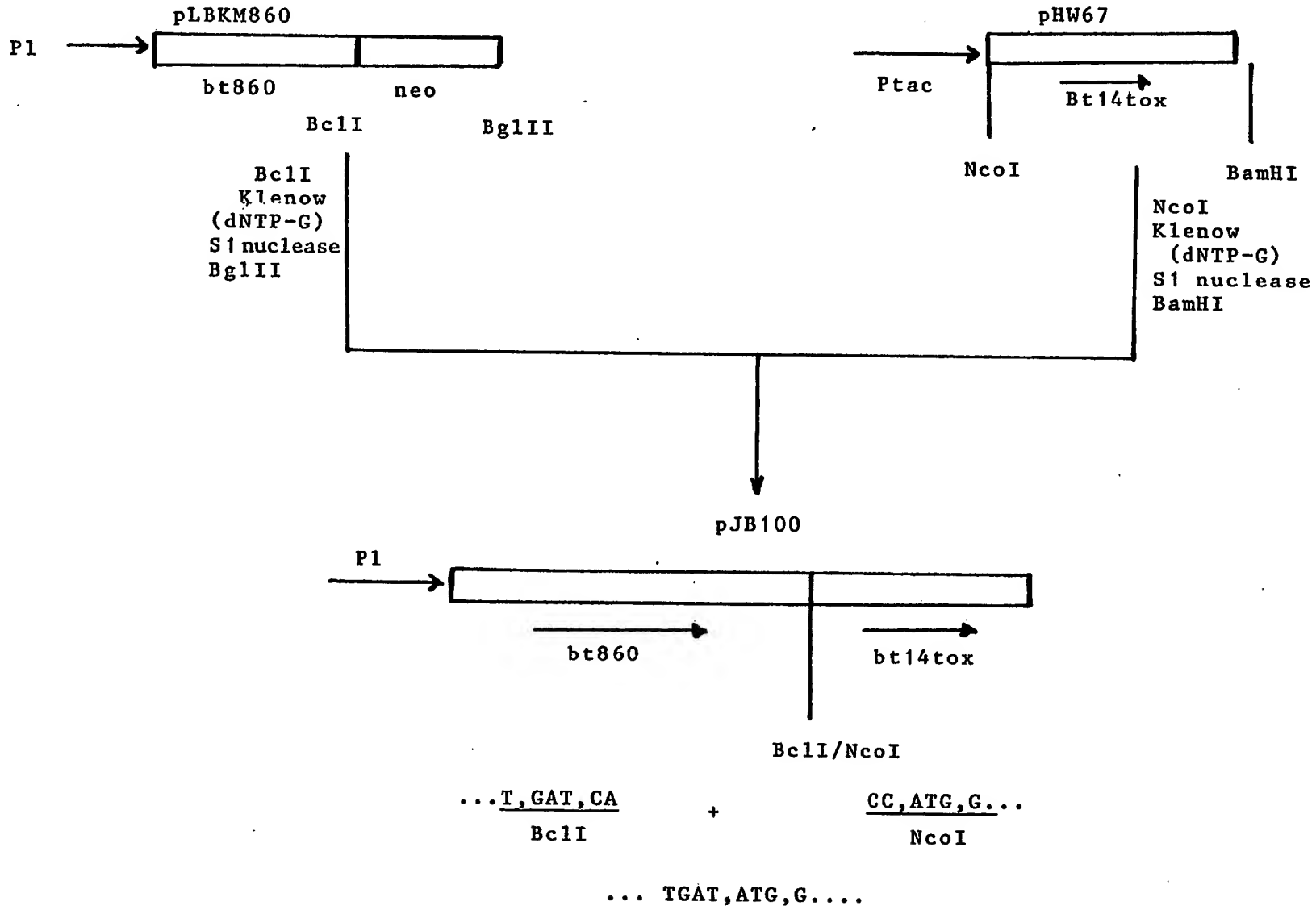


FIG 16-END

Figure 17



EP 0 400 246 A1

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 89 40 1499

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	EP-A-0 228 838 (MYCOGEN CORP.) * whole document; in particular page 7, example 5 *	1,13-16,27,29	C 12 N 15/32 A 01 N 63/00
Y	WO-A-8 808 880 (ECOGEN, INC.) * abstract; page 11, line 18 - page 13, line 12; page 25, line 9 - page 27, line 15; page 41, line 33 - page 42, line 28; claims *	1,13-16,27,29	
D,A	EP-A-0 193 259 (PLANT GENETIC SYSTEMS N.V.) * whole document *	1,13-16,27,29	
Y	EP-A-0 192 319 (MYCOGEN CORP.) * whole document; in particular page 6, lines 20-24 *	1	
Y	EP-A-0 221 024 (SANDOZ AG) * whole document; in particular page 3, lines 30-34 *	1	
A	PATENT ABSTRACTS OF JAPAN vol. 12, no. 391 (C537)(3238), 18 October 1988; & JP - A - 63 137684 (SUMITOMO CHEM. CO. LTD.) 09.06.1988	1	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C 12 N 15/32

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	18-10-1989	JULIA P.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)